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EXPRESSION OF BIOMARKERS BY PERIPHERAL NERVE FIBRES IN HUMAN MASSETER MUSCLE

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Cover illustration: expression of pain biomarkers, namely, SP (blue), NR2B (red), and NGF (baby blue), by nerve fibres (green) associated with myocytes from a microbiopsy taken from the masseter muscle of one female participant after a combined injection of NGF and glutamate into the muscle. The skull presented in the cover was drawn by Johanna Svedenlöf

EXPRESSION OF BIOMARKERS BY PERIPHERAL NERVE FIBRES IN HUMAN MASSETER MUSCLE
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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The thesis will be defended in public at the Department of Dental Medicine, lecture hall 9Q, Alfred Nobels Allé 8, Huddinge, **Friday 19th of February 2021, 13.00 pm**

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إِقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ - خَلَقَ الْإِنْسَانَ مِنْ عَلَق

Learn in the name of the designer - The designer who designed a human from a group of interconnections (595 C.E.)

These are the first two verses revealed to the prophet Muhammad (peace be upon him) by the mighty God.

إهداء إلى من أدين لهم بكل الفضل و الإمتنان أبي محي الدين الحلو
و أمي ثناء عرقسوسي و إلى إخوتي الغاليين غادة ، معتز ، مريم ،
آلاء و أسماء و إلى زوجتي و رفيقتي و حبيبة قلبي ربى
عرقسوسي و ولدي و قرة عيني فيصل

Dedicated to my beloved parents, sisters, and brother, and most of all, my wife, Ruba, and my son, Faisal.

POPULAR SCIENCE SUMMARY OF THE THESIS

Chronic facial pain is a significant problem affecting people's quality of life. Approximately 70% of individuals seeking treatment for facial pain also suffer from pain related to the masticatory muscle, and most of them are women. There is currently no effective treatment for masticatory muscle pain due to the lack of knowledge related to its underlying mechanisms. However, scientists have proposed different factors to contribute to masticatory muscle pain, such as social, behavioural, biological, and psychological factors.

Regarding biology, experimental animal studies have suggested specific pain receptors to be involved in masticatory muscle pain and sex-related differences. However, it is unknown if these findings are applicable to humans as well. Nevertheless, scientists have previously developed experimental pain models that can produce similar pain characteristics to chronic masticatory muscle pain in humans. This idea was adopted by the current thesis to investigate the change in the pain receptors of nerve fibres after experimental induction of pain to the human masticatory muscle.

The thesis results are consistent with animal results, with pain induction succeeding in increasing the expression of pain receptors. Moreover, the results showed that the higher the expression of these receptors by nerve fibres, the higher the pain perception in humans is. Further, pain receptors were found to have higher expression in women when compared to men. Finally, expression of pain receptors increased after pain induction in women, but not men.

The significance of the current findings depends on whether these pain receptors are also found to have higher expression in individuals suffering from chronic masticatory muscle pain. If so, the present results will be the base for an improved diagnosis and/or treatment approaches for patients, potentially fostering the development of medication capable of blocking these pain receptors, benefitting both clinicians and patients.

ملخص الأطروحة العام

حقائق عامة:

- آلام الوجه المزمنة مشكلةٌ كبيرةٌ تؤثر تأثيرًا بالغًا في حياة الذين يعانون منها.
- نحو (٧٠ ٪) من المرضى الذين يسعون للعلاج من آلام الوجه، يعانون من آلام في عضلات المضغ أيضًا.
- معظم الذين يعانون من هذه الآلام من النساء.
- لا يوجد -حاليًا- علاج فعال لألم عضلات المضغ بسبب نقص المعرفة المتعلقة بآلياته الأساسية. إلا أن العلماء أشاروا إلى عوامل مختلفة قد تسبب آلام عضلات المضغ، كالعوامل الاجتماعية والسلوكية والبيولوجية والنفسية.

أسباب الألم ومنهجية البحث:

- أشارت الدراسات التجريبية على الحيوانات، إلى وجود مستقبِلات ألم في الألياف العصبية، مسؤولة عن الآلام المتعلقة بعضلات المضغ، ومسؤولة عن الاختلافات في الإحساس بالألم بين الجنسين. لكن، من غير المعروف ما إذا كانت هذه النتائج قابلة للتطبيق على البشر أيضًا.
- ابتكر العلماء نماذج تجريبية يمكن تطبيقها على البشر الأصحاء، لإحداث ألم مماثل لآلام عضلات المضغ المزمن عند المرضى.
- نحن في هذه الأطروحة عملنا على المبدئ نفسه، حيث أحدثنا ألمًا صناعيًا لعضلة الفك لدى أشخاص سليمين، ثم أخذنا عينات منهم، لدراسة ما إذا كانت مستقبِلات الألم المسببة لآلم عضلات المضغ (التي اكتُشفت سابقًا عند الحيوانات) موجودة عند البشر أيضًا.

نتائج الأطروحة:

- تتوافق نتائج الأطروحة مع نتائج الدراسات التي سبق أن أجريت على الحيوانات، فقد نجح تحريض الألم الصناعي في زيادة نشاط مستقبِلات الألم في الألياف العصبية لدى عضلة الفك عند البشر.
- أظهرت النتائج أنه كلما زاد نشاط هذه المستقبِلات في الألياف العصبية، زاد الإحساس بالألم لدى البشر.
- كذلك وجدت الأطروحة أن مستقبِلات الألم هذه نشطة أكثر عند النساء مقارنة بالرجال.

أهمية النتائج:

- أظهرت نتائج الأطروحة أن مستقبِلات الألم الخاصة بعضلات المضغ التي اكتُشفت سابقًا عند الحيوانات موجودة لدى البشر، وأنها تنشَط عند تعرّض الإنسان السليم للألم الصناعي.
- في حال وجدت هذه المُستقبِلات نشطة -كذلك- عند الإنسان المصاب بآلام عضلات المضغ المزمن، فإن هذا سيكون نقطة انطلاق لتشخيص المرضى، وإيجاد طرق علاجية لهم، كإيجاد أدوية تعمل على إخماد نشاط مستقبِلات الألم هذه، مما قد يخفف من آلامهم.

ABSTRACT

Background: Temporomandibular disorders (TMDs) often manifest as masticatory muscle pain (myalgia). Nerve growth factor (NGF) and glutamate injection into healthy human masseter muscle induced signs and symptoms mimicking those of TMD myalgia and have therefore been suggested as pain models to study the neurobiological mechanisms of this type of chronic pain conditions. Previous animal studies have demonstrated that certain pain biomarkers are involved in the development of pain and mechanical muscle sensitisation induced by NGF and/or glutamate or by inflammation. Examples of such biomarkers are substance P (SP), N-methyl-D-aspartate receptor (NMDA), and NGF. However, it is unknown if NGF and/or glutamate-induced masseter muscle pain and sensitisation in humans share the same pain-pathways suggested to be involved in animals. Therefore, the main aim of this thesis was to investigate the effect of NGF and/or glutamate on the expression of putative pain biomarkers by nerve fibres within the human masseter muscle.

Materials and methods: The thesis consisted of two experiments involving injection of NGF and/or glutamate into a pain-free masseter muscle. Participants included 60 healthy volunteers (30 in each experiment). For the first experiment, sterile NGF (0.4 ml, 25 µg/ml) was injected into the left masseter muscle (experimental side). On the other hand, in the second experiment, 0.2 ml of sterile glutamate (1.0 M) was injected in a muscle that was pre-treated with NGF. For both experiments, microbiopsies were obtained from the right masseter muscle (contralateral side, i.e., baseline) as well as from the experimental side. Moreover, pain characteristics were assessed before and after the injections. Biopsy sections were analysed via immunohistochemistry, where PGP9.5 was used to identify nerve fibres, while primary antibodies against each substance and their corresponding secondary antibodies were used to identify the putative biomarkers of interest. Sections were visualised with a confocal microscope (Leica TCS SPE).

Results: NGF administration alone did not cause an increase in the frequency of nerve fibre expression ($P>0.05$). In contrast, the combined injection of NGF and glutamate increased the expression of SP alone ($F=13.713$, $P=0.002$), with NR2B ($F=10.599$, $P=0.006$) or with NGF ($F=5.151$, $P=0.040$), and all together ($F=4.774$, $P=0.046$). This increase was also greater in women than in men ($P<0.05$). Pain characteristics correlated positively with the expression of NR2B alone or together with SP by nerve fibres ($P<0.05$). In other words, a greater expression of NR2B by nerve fibres or by putative afferent fibres (expressing SP) was associated with increased pain.

Conclusion: It appears that, in humans, muscle pain occurrence and sensitisation depend significantly on peripheral presumptive afferent fibres expressing NMDA-receptors and NGF. It also appears that the expression by these afferent fibres account for variation in pain characteristics between males and females in the context of experimental induction of myalgia. Nevertheless, additional research must be conducted to determine whether such findings are related to TMD myalgia mechanisms.

LIST OF SCIENTIFIC PAPERS

I. Density of nerve fibers and expression of substance P, NR2B-receptors, and nerve growth factor in healthy human masseter muscle: An immunohistochemical study
Abdelrahman M. Alhilou*, Akiko Shimada, Camilla I. Svensson, Malin Ernberg, Brian E. Cairns, Nikolaos Christidis. 08 October 2020. Journal of oral rehabilitation, https://doi.org/10.1111/joor.13109*

II. Sex-related differences in response to masseteric injections of glutamate and nerve growth factor in healthy human participants
Abdelrahman M. Alhilou, *Akiko Shimada, Camilla I. Svensson, Peter Svensson, Malin Ernberg, Brian E. Cairns, Nikolaos Christidis.*
Submitted manuscript

III. Functional Change in Experimental Allodynia After Glutamate-Induced Pain in the Human Masseter Muscle
Akiko Shimada, ***Abdelrahman M. Alhilou,*** *Peter Svensson, Malin Ernberg, Nikolaos Christidis. 23 November 2020, Front.Oral.Health, https://doi.org/10.3389/froh.2020.609082*

IV. Injection of nerve growth factor and glutamate increase the density and expression of afferent nerve fibers in the healthy human masseter muscle
Abdelrahman M. Alhilou*, Akiko Shimada, Camilla I. Svensson, Peter Svensson, Malin Ernberg, Brian E. Cairns, Nikolaos Christidis.*
Submitted manuscript

CONTENTS

LIST OF ABBREVIATIONS

Aδ & Aβ	A-delta and A-beta fibers.
CNS	Central nervous system
DC/TMD	Diagnostic criteria for temporomandibular disorders
DRG	Dorsal root ganglion
eVAS	electronic visual analogue scale
EXP	Experimental assessments of pain induced characteristics
GAD	Generalised Anxiety Disorder
IASP	International Association for the Study of Pain
IHC	Immunohistochemical analysis
JFLS	Jaw Functional Limitation Scale
JFLS	Jaw functional limitation scale
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NRS	Numeric rating scale
NRS	numeric rating scale
OHIP	Oral health impact profile
PAG	Periaqueductal gray matter
PBS	Phosphate-buffered saline
PHQ	Patient History Questionnaire
PPT	Pressure pain thresholds
PRI	Pain Rating Index
PSS	Perceived Stress Scale
RPE	Borg’s Rating of Perceived Exertion scale
RVM	Ventromedial medulla
SP	Substance P
TG	Trigeminal ganglion
TMD	Temporomandibular disorders
TMJ	Temporomandibular joint
V	Trigeminal nerve
5-HT3	5-hydroxytryptamine receptor type 3

1 INTRODUCTION

Chronic pain represents a health issue of global prevalence (Breivik et al. 2006; Tsang et al. 2008). Moderate to severe chronic pain affects around 20% of adults, causing personal distress and having significant economic implications at societal level (Breivik et al. 2006). In Sweden, the average yearly cost in 2008 was estimated to be €32 billion (Gustavsson et al. 2012), while the annual cost in 2010 in Denmark and the United States was respectively €17.8 billion and €635 billion (Christensen et al. 2011; Gaskin and Richard 2012).

Various body parts can be subject to chronic pain. The type of chronic pain affecting the orofacial region most frequently is temporomandibular disorders (TMD) (Dworkin et al. 1990). TMD encompasses various different issues associated with chronic pain that affect the temporomandibular joint, the masticatory muscles or both (Dworkin et al. 1990; Romero-Reyes and Uyanik 2014). There is evidence that female individuals are more prone to TMD compared to male individuals (LeResche 1997). According to epidemiological studies, as many as two out of three patients with TMD are women (Isong et al. 2008). Besides being disagreeable from a sensory perspective, as for other chronic pains, TMD pain can also be emotionally detrimental, causing sufferers to feel anxious, stressed, unable to sleep well, miserable, guilty, and isolated, which could ultimately lead to development of depression (Rai and Kaur 2013; Reissmann et al. 2014; Thomas 2000). This often leads to increased work absence due to sickness, increased consumption of painkillers, and increased necessity of care, which results not just in reduced quality of life for the individuals suffering from chronic pains, but also in increased costs to society (Barros Vde et al. 2009; Hallberg and Carlsson 2000; Ohman et al. 2003; Thomas 2000; Von Korff et al. 1990).

Ten to 15% of the general population and approximately 70% of individuals with TMD suffer from masticatory muscle pain (myalgia). TMD myalgia is known as muscle-related pain of a dull and aching nature that is influenced by jaw mobility, function or parafunction. It is the main source of pain in a quarter of TMD cases (Bertoli et al. 2018; Cairns 2010; Manfredini et al. 2011; Marklund and Wanman 2008; Schiffman et al. 2014). Hence, it can be considered the most common subtype of TMD.

It is believed that there are multiple interlinked causes of different origin (biological, psychological, social, and environmental) that underpin the occurrence of TMD myalgia (Greene 2010; Svensson and Kumar 2016). With regards to biological factors, some studies have suggested micro-inflammation to be involved in TMD myalgia, in which algogenic substances activate or sensitise nociceptive free nerve endings, thereby causing pain (Graven-Nielsen and Mense 2001; Mense 1993; 1999; Okeson 2014). However, generalised pain conditions such as fibromyalgia (Clauw and Crofford 2003; Henriksson 2003), as well as the majority of TMD, do not show signs of inflammatory changes (Singer and Dionne 1997; Zarb et al. 1995). Consequently, there is a possibility that TMD myalgia may occur as a result of distinct receptor mechanisms (Lam et al. 2005). TMD myalgia-related presumptive pain biomarkers (e.g. N-methyl-D-aspartate (NMDA) receptors, serotonin receptors, nerve growth

factor (NGF), and substance P (SP)) have not been extensively researched (Cairns et al. 2003; Castrillon et al. 2010; Christidis et al. 2014; Ernberg et al. 2000a; 2000b; Stohler 1997; Sung et al. 2008; Wong et al. 2014). The pathophysiological mechanism initiating and maintaining TMD myalgia and the basis of female predominance are not well understood yet. Hence, it is important to further expand the neurobiological knowledge behind it. In the long run, this could, in turn, be a base for improving diagnosis and/or treatment approaches.

2 LITERATURE REVIEW

2.1 PAIN: A HISTORICAL BACKGROUND

Pain has existed since the dawn of humanity. The word pain originates from the Greek word "Poine", which means "penalty". Therefore, in ancient times, suffering from pain was interpreted as a punishment from God. Throughout history, humans have had different beliefs about pain. For example, Ancient Egyptians believed in the tribal concept that pain existed outside the body and resulted from an "intrusion", whereas the early Native Americans considered the heart as the organ responsible for the pain sensation. The Greek philosopher Aristotle (384–322 BC) also believed that the heart was the centre for pain processing. In contrast, the Greek philosopher Stratton and Herophilus and Eistratus from Egypt suggested that the brain was the centre of pain (Parris and Johnson 2014). Galen reemphasised the concept of the central nervous system suggested by the Egyptian philosophers and developed terms for pain sensation (Ochs 2004). The Muslim physician Ibn Sina (Avicenna in Latin) extended Galen's pain terminology and described pain in terms similar to those used nowadays in the McGill Pain Questionnaire (Tashani and Johnson 2010). The Ancient Greek were the first who believed that pain was a sensory function stemming from peripheral stimulation. The first reasonable pain theory was put forth by Descartes, who argued that pain was a perception occurring in the brain and distinguished sensory transduction, which is a neural phenomenon currently termed nociception, from pain as perceived experience (Descartes R 1664). Several other theories have contributed to a general understanding of pain and its mechanisms. Until recently, the most popular pain definition was the one written by the International Association for the Study of Pain (IASP) in 1979 "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage". In July 2020, the same association modified the definition to become the following "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (Raja et al. 2020).

2.2 CLASSIFICATION OF PAIN

Pain can be classified in different ways. It can be categorised according to its duration, where if it persists for a limited time due to normal response to a noxious stimulus it will be considered acute, while if it persists for longer than the normal course of a condition or its healing phase (usually 3-6 months) it will be recognised as chronic or persistent pain. Pain can also be classified according to the body parts affected, such as pain in the head, mouth or face, which can be sub-classified into organs involved in pain, for example, musculoskeletal, subcutaneous, visceral, nervous, and so on. Another important classification is the aetiology of pain (inflammatory, genetic, congenital or psychological). Furthermore, it can be classified based on its temporal characteristics (intermittent, continuous or single episode) (Miles et al. 2004). Pain can also be classified according to its underlying mechanism by drawing on the increasing understanding of the molecular and cellular mechanisms through which pain occurs (Woolf et al. 1998). Damage to tissues other than neural that lead to pain due to nociceptors activation is

called nociceptive pain. Neuropathic pain, otherwise known as a lesion or disease in the peripheral nervous system and/or central nervous system (CNS). Finally, pain emerges from an altered nociceptive function with neither clear signs of tissue damage nor signs of neural disease or lesion are known as nociplastic pain (IASP Taxonomy 2017).

2.3 MECHANISM OF PAIN

In the human body, stimuli of pain are detected by pain or algesic receptors known as nociceptors, which then issue signals to the relevant brain centres. Different types of nociceptors can transmit different kinds of painful stimuli. Mechanoreceptors, for example, transmit strong mechanical stimuli caused by high pressure. Thermoreceptors transmit signals about stimuli caused by changes in temperature. Chemoreceptors mediate changes in the chemical environment and are important in inflammation. Polymodal receptors are capable of transmitting signals related to multiple forms of stimuli (Graven-Nielsen et al. 2008; Okeson 2013). The nociceptors transmitting signals about pain stimuli from the peripheral to the central nervous system are called afferent nerve fibres. However, other nerve fibres can also be afferent but convey information unrelated to pain, such as pressure, touch, and vibration, to the central nervous system. According to the Lloyd classification (Lloyd 1943), these fibres are mostly from type II fibres and are known as Aβ (A-beta fibres), which are myelinated and large in diameter. On the other hand, afferent fibres transmitting pain stimuli are mainly from type III (Aδ fibres) and IV (C fibres). Aδ afferent fibres are thin and myelinated fibres that convey an intense, sharp, and well-defined pain, while the C fibres are thinner and unmyelinated and convey a more abrasive, indirect pain (Graven-Nielsen et al. 2008; McMahon et al. 2013; Mense 1993).

In the face, the pain is transmitted peripherally to the brain via the fifth cranial nerve, known as the trigeminal nerve (V) (Figure 1, A). The V ganglion contains the cell bodies of primary afferent fibres, the axons of which display central projection to the peripheral tissues and to the brain stem. The brain stem represents the site where the signals about the pain stimuli switch to the CNS. The major elements of the V brain stem system are the V primary sensory nucleus and the V spinal tract nucleus. In the V brain stem, the signal is passed on to the subnucleus caudalis (a subdivision of the V spinal tract nucleus), which then transmits the information to higher parts in the brain via a secondary order. The laminated structure exhibited by the subnucleus caudalis shares similarities with the spinal cord dorsal horn. The thalamus is the subsequent site where signal switch and are transmitted through tertiary neurons going to the cerebral cortex, where pain recognition eventually occurs (Graven-Nielsen and Mense 2001; McMahon et al. 2013; Sarnat and Laskin 1992) (Figure 1, B).

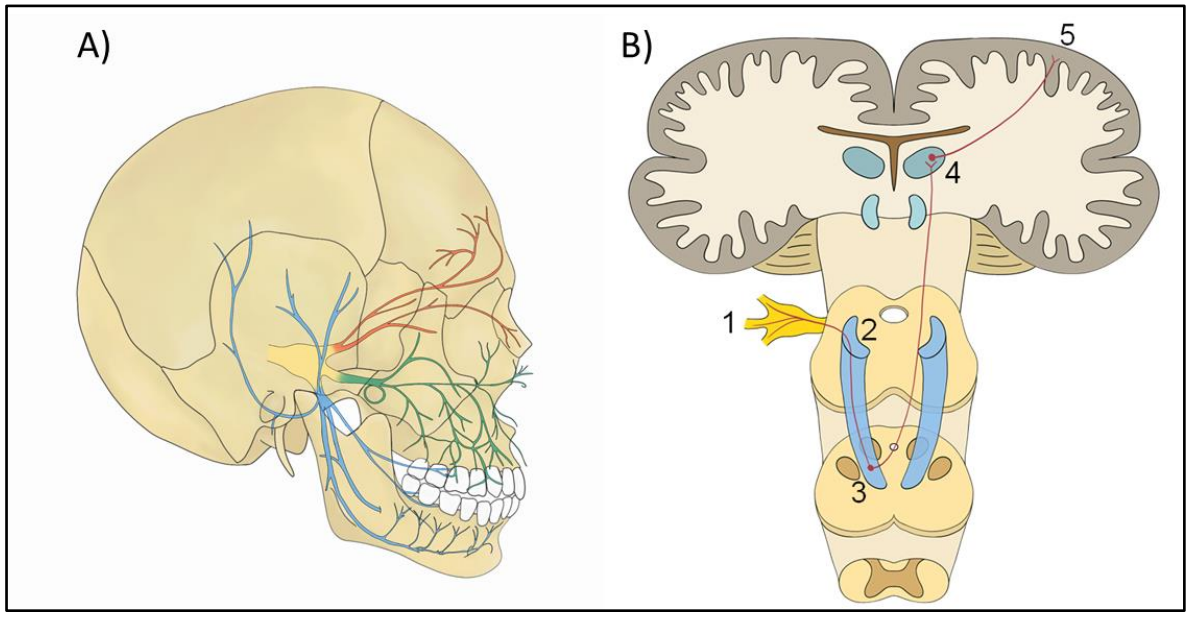

Figure 1. A) Illustration of the extension of the trigeminal nerve and its branches. The trigeminal ganglion is divided into three branches. The upper (red) is the ophthalmic nerve (V: 1), the middle (green) is the maxillary nerve (V: 2), and the lower (blue) is the mandibular nerve (V: 3) in which one of its branches (masseteric nerve) innervates the masseter muscle. **B)** Schematic illustration of how pain signals are transmitted from the trigeminal ganglia (1) to the sensory complex (2) of the brain stem to be connected to the subnucleus caudalis (3). In the subnucleus caudalis, the signal is switched via secondary neurons to the thalamus (4). Tertiary neurons in the thalamus transmit the signal to the cortex (5; cerebral cortex), where the pain is finally perceived. Figure 1 is adapted from illustrations from the book "Bettfysiologi – orofacial smärta och käkfunktion" (Nikolaos Christidis 2020) with permission from the publisher.

2.3.1 Pain modulation

The body can amplify or reduce signals transmitted to the brain (pain modulation) in different ways. Two mechanisms are known to slow down the pain signals, namely, the gate-control theory and the descending pain suppression pathway.

Patrick Wall and Ronald Melzack first suggested the gate-control theory in 1965. The theory got its name from the way it has been illustrated in the literature, i.e. gates being opened and closed depending on the size of the nerve fibres being activated (Moayedi and Davis 2013). For example, non-painful signals can be activated to block pain transmission from the dorsal root ganglion up to the cortex. Cold stimulus application, electrical nerve stimulation, acupuncture or massage are some methods used to inhibit transmission of pain (McMahon et al. 2013).

The descending pain suppression pathway depends on the body's own analgesic substances that slow down pain signals. The pathway inhibits incoming pain signals at the spinal cord. The periaqueductal grey matter (PAG) and the rostral ventromedial medulla (RVM) facilitate the downward projection of the pathway to exert its action directly on the spinal dorsal horn or the subnucleus caudalis for the peripheral and central nervous system, respectively, with release of serotonin and norepinephrine. Conversely, the indirect pathway involves activation of

inhibitory interneurons, which in turn release enkephalin (Fields 2000; Hadjipavlou et al. 2006; Millan 2002).

On the other hand, amplification of signals transmitted to the brain is known as central sensitisation and entails reduction in inhibition at the spinal cord and increase in events at the synaptic level (Woolf 2011). The changes that neuronal characteristics and functions undergo at the brain level are termed by neuroscientists as neuroplasticity. Central sensitisation has two main features, namely, allodynia and hyperalgesia. The former refers to perception of pain to a non-painful stimulus, while the latter is an increase in sensitivity to a painful stimulus (Miles et al. 2004).

2.4 SKELETAL MUSCLE (PHYSIOLOGY AND ANATOMY)

The skeletal muscle represents almost half of the body weight and is attached to bone by tendons to allow body movement when the muscles contract. Unlike the other two types of muscles (smooth muscle and cardiac muscle), which are under the control of the autonomic nervous system, the skeletal muscle is under voluntary control (McCuller and Callahan 2019; Ostrovidov et al. 2014).

Muscle and nerve fibres, blood vessels, and connective tissue are among the constituents of skeletal muscle. Three types of connective tissue are found in skeletal muscle (Figure 2). The first type, known as epimysium, is dense, irregular, and surrounds the whole muscle. This sheath of connective tissue maintains the structural integrity of the muscle during contraction. Each skeletal muscle is made up of several bundles called fascicle, which is surrounded by the second type of connective tissue known as perimysium. A number of 10-20 ordered muscle fibres make up the fascicle and are in turn covered by an additional type of connective tissue, namely, the endomysium. Every muscle fibre constitutes one cell with more than one nucleus (McCuller and Callahan 2019). A muscle fibre contains many myofibrils, which are the threads that contract the muscle fibre (Dave and Varacallo 2018). The myofibrils consist of several segments called sarcomeres and are separated by Z-lines, which gives striated muscles their characteristic appearance. Each sarcomere contains thin filaments (actin) and bundles of thick filament known as myosin are loosely present between the actin strands. Through a process called the cross-bridge cycle, actin and myosin can then "climb" on each other and thus cause sarcomeres, and implicitly the whole muscle, to contract (Huxley and Hanson 1954; Ostrovidov et al. 2014).

The muscle fibres are innervated by nerve fibres that conduct nerve impulses outward from (motor) and inward to (sensory) the CNS. When the motor nerve is activated or stimulated, the axon terminals release acetylcholine, a neurotransmitter that binds to its receptors in the motor end-plate at the muscle fibres. This in turn initiates a depolarisation of the muscle fibres, causing muscle contraction (Slater 2017). Muscle spindles are sensory receptors within the muscle specialised to detect changes in the intrafusal fibres within the muscle (e.g. stretching), which in turns send this information (i.e. changes in the length of fibres) to the CNS (Okeson

2013). Two types of afferent nerve fibres can supply the muscle spindles, namely, the fast thick myelinated Aα and Aβ fibres (Okeson 2013).

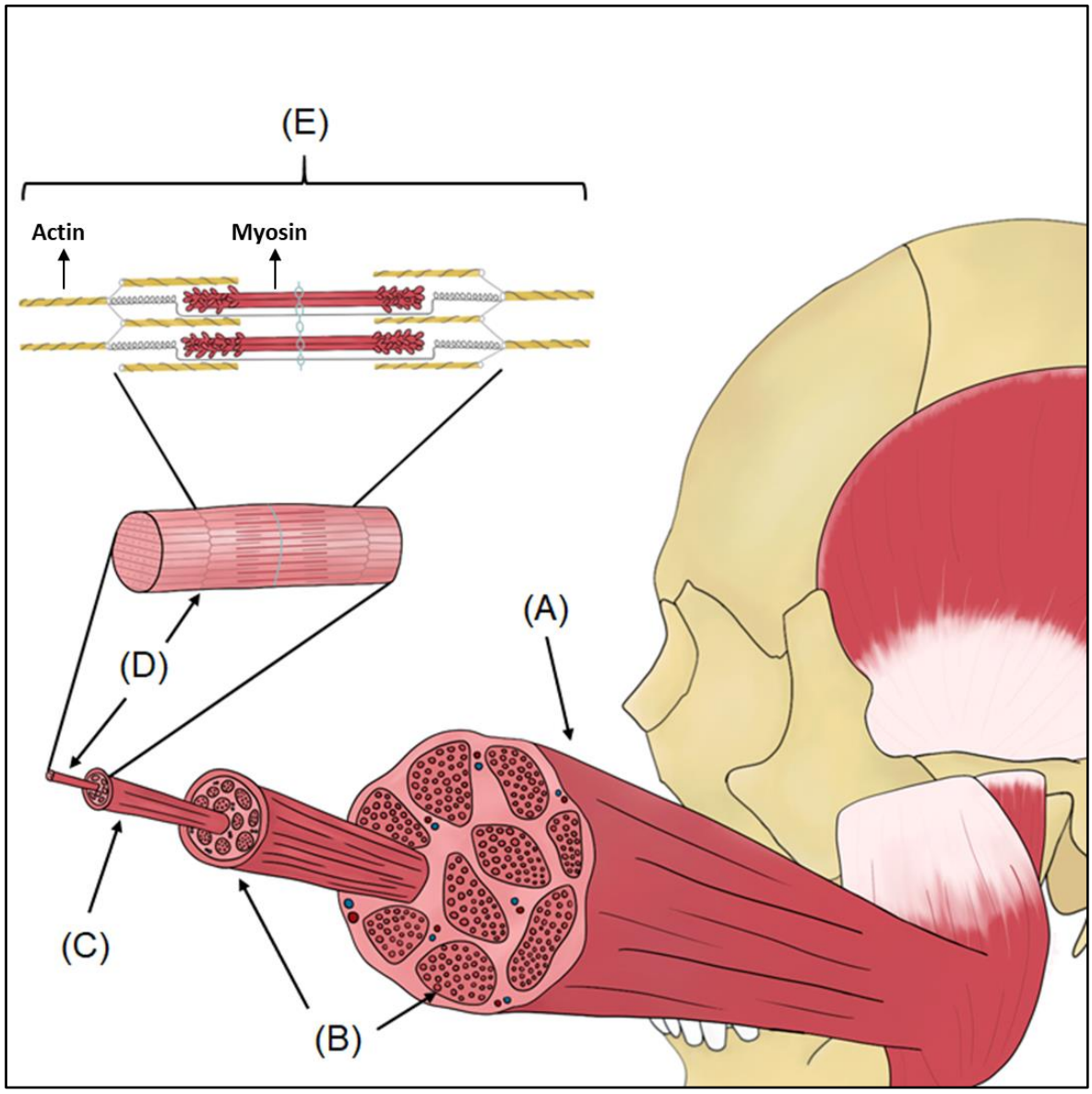

Figure 2: Illustration of structures within the masseter muscle, showing **(A)** the layer of connective tissue (epimysium) surrounding the whole muscle body, **(B)** a muscle fascicle surrounded by perimysium (pinkish area in between fascicles), **(C)** a muscle fibre inside the fascicle, **(D)** a myofibril within the muscle fibre surrounded by endomysium, and **(E)** a sarcomere within a myofibril composed of actin (thin filament) and myosin (thick filament)**.** Figure 2 is a copy from the book "Bettfysiologi – orofacial smärta och käkfunktion" (Nikolaos Christidis 2020) with permission from the publisher.

2.4.1 Muscle of mastication

The muscles responsible for the exact movements of opening and closing of the mandible are known as mastication muscles and are found on each side of the cranium. The mouth-closing jaw muscles are the masseter, temporalis, and medial pterygoid, while the mouth-opening muscles are the digastric and lateral pterygoid (Okeson 2013).

2.4.2 Masseter muscle

The quadrangle-shaped masseter is a robust muscle comprising a deep portion and a superficial portion. The deep portion begins from the inferior edge of the zygomatic arch and attaches to the external surface of the mandibular ramus. The superficial part is situated over the deep part and originates more anteriorly at the maxillary process of the zygomatic bone and inserts in the masseteric tuberosity. The masseter muscle can elevate the mandible and close the mouth due to muscle contraction. It can also protrude the mandible allowing for anterior movement of the jaw. The masseter muscle gets its vascular supply from a branch of the maxillary artery known as the masseteric artery, while it gets its nerve supply from an extension of the mandibular division (V3) of the V nerve called the masseteric nerve (Corcoran and Goldman 2020).

2.5 PATHOPHYSIOLOGY OF TMD MYALGIA

Although little is known about the pathophysiological mechanisms involved in TMD myalgia, different theories have been proposed. For example, pain in the jaw muscle may result from an excessive reflex tone (a physiological reflex to protect the injured area) in response to pain in the temporomandibular joint (TMJ) (Tanaka et al. 2008). The vicious cycle theory is another theory that has been suggested, maintaining that pain induced by muscle tone can cause muscle spasm and fatigue, which in turn can further increase pain and so on (Murray and Peck 2007). Additional theories include the pain adaptation model and the integrated pain adaptation model. Both theories can be construed as refinement of the jaw-reflex theory, with the exception that the latter considered the interindividual variations in motor strategies to control pain (Cairns 2010). Other researchers believe that the muscle itself can be the primary source of pain. For example, tender non-masticatory muscles appear to have regions with an increased level of neuropeptides and cytokines as well as decreased PH level, which are signs of inflammation (Shah et al. 2008). Hypoxia or ischemia induced in the masseter muscle by bruxism or excessive teeth grinding has also been shown to contribute to muscle fatigue and pain (Monteiro and Kopp 1989). Furthermore, it has recently been proposed that chemical or mechanical noxious stimuli applied to the masticatory muscle can elevate algogenic substances and neuropeptides in the area affected, which in turn bind to their specific receptors on afferent nerve fibres and function as polymodal nociceptors (Cairns 2010).

2.6 ALGOGENIC SUBSTANCES AND PERIPHERAL RECEPTORS

As mentioned in the previous paragraph, the human masticatory muscle can be affected directly or indirectly by endogenous substances (neurotransmitters) modulating pain through the activation of specific receptors. The following section will describe some of these neurotransmitters and their corresponding receptors.

2.6.1 Glutamate

Besides being a common non-essential amino acid, glutamate also serves as an important bioenergetic substrate for both normal and neoplastic cell proliferation. Additionally, glutamate fulfils the function of excitatory neurotransmitter and by activating its own receptors it participates directly in signalling pathways. Furthermore, glutamate can activate both types of excitatory amino acid receptors (Willard and Koochekpour 2013), namely, inotropic and metabotropic receptors. In turn, inotropic receptors can be classified into three subgroups, namely, one NMDA receptor and two non-NMDA receptors (Collingridge and Lester 1989; Dingledine and Conn 2000; Kew and Kemp 2005; Monaghan et al. 1989; Neugebauer 2002). The NMDA receptor represents a channel of mixed cations permitting Na+, K+, and Ca+ to pass through (Kew and Kemp 2005; Paoletti and Neyton 2007). Its composition encompasses NR_1, NR_2 (A, B, C, D), and NR_3 (A, B) subunits combined in different ways (Bleakman et al. 2006; Gogas 2006; Kew and Kemp 2005; Paoletti and Neyton 2007). Despite the tetrameric structure, the functional receptors comprise NR_1/NR_2 subunit dimers. Moreover, the receptor may be less conductive and permeable to calcium than the NR_1/NR_2 receptor because of the possibility of substitution of one of the NR_2 subunits with an NR_3 subunit (Bleakman et al. 2006; Gogas 2006; Kew and Kemp 2005; Paoletti and Neyton 2007).

The presence of NMDA receptors has been reported in the dorsal root (DRG) and the trigeminal ganglion (TG) (Sahara et al. 1997), as well as on small diameter primary afferents in rats (Coggeshall and Carlton 1998). It has been shown that the subunit 1 of the NMDA receptors is present in painful human Achilles tendons (Alfredson et al. 2001). The NMDA receptor subunit type 2B (NR2B) has been shown to be expressed by masseter muscle peripheral nerve fibres in healthy participants (Wong et al. 2014).

By activating peripheral NMDA receptors, glutamate may trigger nociceptive reactions and muscle pain (Cairns et al. 2002; Cairns et al. 2006; Cairns et al. 2003). One study reported that, compared to men, women felt greater pain when glutamate was injected in the masseter muscle (Cairns et al. 2001; Svensson et al. 2003b). In rats, this phenomenon was attributed to the fact that NMDA receptors were expressed at a higher level, which was correlated to levels of oestrogen in human females (Dong et al. 2007). However, it is still unknown if activation of peripheral NMDA receptors underlies the pain sex-related differences in healthy subjects or in patients with chronic local myalgia.

2.6.2 The neuropeptide Substance P (SP)

SP is a peptide that plays different physiological roles in the body, including pain. Neurokinin-1 receptor (a specific receptor for SP) has been found expressed in non-neuronal cells, which can further indicate that SP is involved in functions other than pain. SP is expressed by different cells in the body such as endothelial cells, epithelial cells, neurons, and many others (Mashaghi et al. 2016).

The well-known pathophysiological roles of SP are related to inflammation as well as nociception. SP can activate both the adaptive and innate immune cells and vice versa, which in turns provides insight into the important role played by SP in inflammation (Ho et al. 1997; Marriott and Bost 2000). SP can modulate nociception through the activation of SP receptor neurokinin-1 (Gautam et al. 2016; Mense 2003; Schank and Heilig 2017; Teodoro et al. 2013). SP containing neurons comprises 20-25% of total DRG and TG neurons (Hokfelt et al. 1975), in which half of these neurons (50%) are C-fibre and 30% are Aδ-fibre neurons (McCarthy and Lawson 1989). After its synthesis in the DRG, SP is transported to the peripheral afferent terminals (Cuello et al. 1978) to be expressed (Cuello et al. 1978; Reinert et al. 1998). However, the SP role at the peripheral end of the nerve fibres in muscle sensitisation has not been extensively investigated.

2.6.3 The neurotrophin nerve growth factor (NGF)

Neurotrophins are a family of related proteins that play an essential role in neuronal differentiation, survival, growth, and apoptosis. They are also involved in nociceptive transmission. They are divided into five types, which are NGF, brain-derived nerve growth factor, neutrophin 3 (NT-3), NT-4, and NT-5. NGF can be produced by different types of cells, such as neurons, endothelium, smooth muscles, mast cells, eosinophils, and lymphocytes (Aloe et al. 2015).

Tyrosine kinase A (TrkA) receptor and 75-kDa neurotrophin receptor ($p75^{NTR}$) with high and low affinity, respectively, are the two distinct membrane-bound receptors that are activated by NGF in the context of the role it plays in nociception and/or inflammation (Apfel 2000; Bennett 2001; Pezet and McMahon 2006; Sung et al. 2018). Moreover, NGF can regulate the expression of neuropeptides by nociceptors centrally (Skoff and Adler 2006).

Many types of chronic muscle pain are associated with high NGF levels (Anand 1995). Therefore, anti-NGF antibodies are considered to be effective for the management of pain related to osteoarthritis and chronic pain affecting the lower back (Kivitz et al. 2013; Lane et al. 2010). Intravenous injection of NGF was reported to cause diffuse myalgia for almost one week, including pain in the masseter muscles (Petty et al. 1994). Local manifestations of hyperalgesia, mechanical allodynia, and pain associated with difficult jaw motion persisting for seven days or more arose when the human masseter muscle was injected with NGF. Such manifestations were nearly identical to those of TMD myalgia. Furthermore, women exhibited a higher level of NGF-triggered sensitisation compared to men. Those results suggested that NGF injection was a valid model to study the pathophysiological mechanism of TMD myalgia

(Svensson et al. 2003a; Svensson et al. 2008a). Research on rats models indicated that muscle sensitisation was the outcome of interaction between NGF and NMDA receptors. Furthermore, nerve fibres displayed higher expression of SP and NGF in female rats than in male rats, which was identified as the reason for the variation between the sexes regarding sensitisation of masseter muscle triggered by NGF (Wong et al. 2014). However, it is still unknown whether such observations are also applicable to humans.

3 RESEARCH AIMS

There is a lack of evidence about the pathophysiological mechanisms that initiate and maintain TMD myalgia, so the main purpose of this thesis was to investigate the cellular and molecular mechanisms of pain mediation and the basis for the female predominance. This can provide an opportunity to improve the diagnostic procedures and, in the long run, lead to new or improved therapeutic approaches.

The specific objectives of the thesis were:

- To examine the nerve fibre density and expression of SP, NR2B, and NGF by the fibre, either on their own or combined, in the masseter muscle of human individuals without health problems, with comparison between myocytes and connective tissue in the muscle body as well as comparison between males and females ***(Study I)***;
- To inject NGF and glutamate on their own or together in the masseter muscle of healthy humans to determine their impact on pain perception, mechanical muscle sensitisation, nerve fibre density, and expression of SP, NR2B, and NGF either on their own or together in the muscle nerve fibres ***(Study II, III and IV)***;
- To determine if there is any correlation between nerve fibre expression and the pain characteristics triggered by the employed experimental models of pain as well as to examine whether those characteristics vary between sexes ***(Study II and IV)***.

The specific hypotheses for the thesis were:

- The nerve fibre density and expression of SP, NR2B, and NGF are greater in nerve fibres within connective tissue than in the nerve fibres associated with myocytes within the masseter muscle. Moreover, the density and expression of nerve fibres are greater in women than men ***(Study I)***;
- Pain, mechanical muscle sensitisation, nerve fibre density and the expression of SP, NR2B, and NGF by the nerve fibre are increased when NGF is injected in the masseter muscle either on its own or alongside glutamate, and this increase is higher in women compared to men ***(Study II, III and IV)***;
- The degree of mechanical sensitisation induced by the injection of NGF alone and/or in combination with glutamate is positively correlated to SP, NR2B, and NGF expression by nerve fibres. There is also a positive correlation between the magnitude of muscle pain induced by glutamate injection in the masseter muscle and NR2B expression by presumptive afferent nerve fibres ***(Study II and IV)***.

4 MATERIALS AND METHODS

The thesis consisted of two experiments where NGF and/or glutamate were injected into the masseter muscle. Based on the type of analysis used, namely, assessment of experimentally-induced pain characteristics and/or immunohistochemical analysis of muscle biopsies, the experiments were divided into four studies (Figure 3).

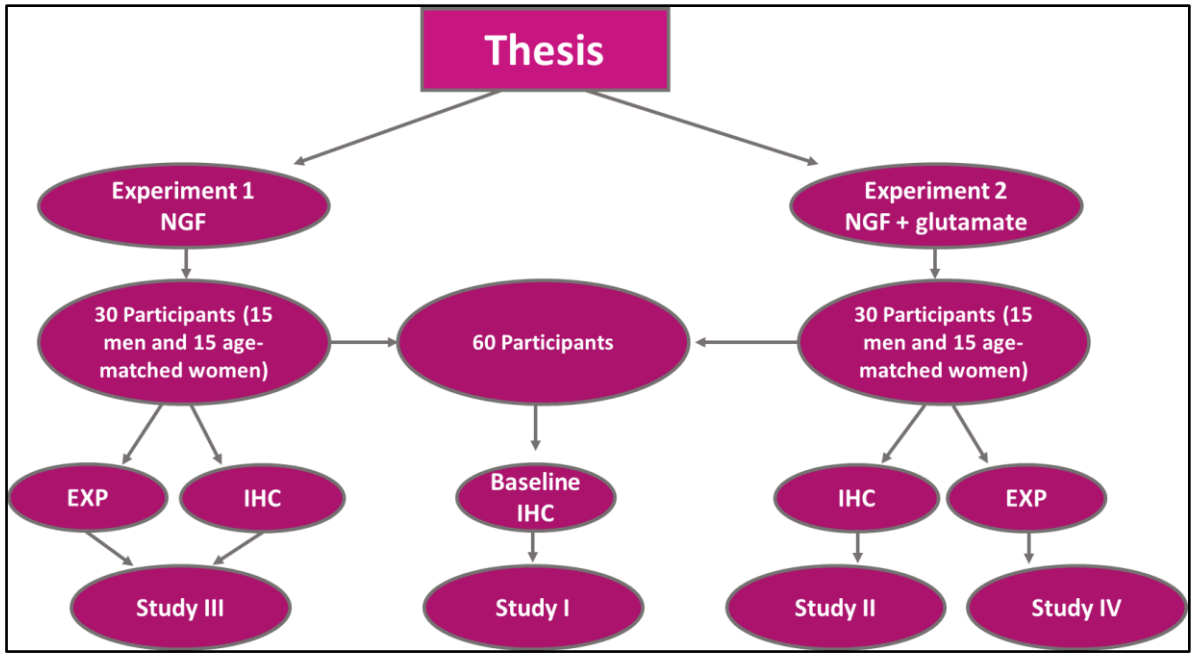

Figure 3: The chart explains how the four studies were devised based on the type of analysis used for experiment 1 (injection of NGF into masseter muscle) and experiment 2 (injection of NGF + glutamate into the masseter muscle). Moreover, it presents the number of participants included in the thesis experiments. For each experiment, two types of analysis were performed: **1)** Assessment of experimentally-induced pain characteristics (EXP) and **2)** Immunohistochemical analysis (IHC). Baseline IHC data (without intervention) were taken from both experiments and were used in ***Study I.***

4.1 PARTICIPANTS

The research sample consisted of 60 voluntary participants who were divided equally between the two sexes and had no health problems. The distribution of participants included in the four studies is presented in Table 1.

Table 1. The distribution in number (n) of participants included in ***Studies I-IV*** and their mean age (years) ± standard deviation. Note that participants in ***Study I*** are the same participants included in ***Studies II, III and IV***.

Study	I	II	III&IV
Healthy participants			
All (n)	60	30	30
Men	30	15	15
Women	30	15	15
Age (years)			
All	28 ± 10	30 ± 12	24 ± 3
Men	28 ± 8	31 ± 12	25 ± 4
Women	26 ± 9	29 ± 12	22 ± 2

Participants were included in the research if they were older than 20 years of age and were healthy, whereas they were not included if they suffered from pain or tenderness on palpation in the face area, systemic inflammatory conditions, neurological conditions, conditions related to whiplash, fibromyalgia, and neuropathic pain, or if they were pregnant, used pharmaceutical agents other than the contraceptive pill long term or used analgesic or anti-inflammatory drugs one day before biopsy. The participants were informed about the research process and their eligibility was assessed during different visits based on the TMD diagnostic criteria (DC/TMD) (Schiffman et al. 2014). The purpose of the clinical exam was to ascertain that the participants did not experience tenderness on touch around the orofacial area that could distort the outcomes.

4.2 DESIGN

Two experiments were conducted, each of which consisted of three sessions, as shown in Figure 4 (A and B) and described in the next part.

The first session (day 0) in both experiments commenced with a standardised clinical examination according to the diagnostic criteria of TMD (DC/TMD) (Schiffman et al. 2014), including jaw movement capacity, pain upon jaw movements, and presence of TMJ sounds as well as palpatory tenderness of the TMJ and jaw muscles. Moreover, participants were asked to fill the DC/TMD Axis II questionnaire, which includes the Symptoms Questionnaire for TMD symptoms, the Jaw Functional Limitation Scale (JFLS) for physical functioning (Ohrbach et al. 2008), the Perceived Stress Scale (PSS-10) (Willert et al. 2009), the Patient History Questionnaire (PHQ-9 and PHQ-15) (Kroenke et al. 2001; 2002), as well as the Generalised Anxiety Disorder Questionnaire (GAD-7) (Spitzer et al. 2006) for assessment of emotional functioning and the Oral Health Impact Profile for life quality (OHIP-14) (Slade and Spencer 1994). Subsequently, some parameters were applied and recorded, such as: **1)** pressure

pain thresholds (PPT) **2)** temporal summation and **3)** functional tests (chewing-induced pain and fatigue).

This was followed by injection of 1 M of glutamate (0.2 ml sterile solution; Skanderborg Apotek, Aarhus, Denmark) in **experiment 1** or 0.4 ml NGF (25 μg/ml sterile solution; Skanderborg Apotek, Aarhus, Denmark) in **experiment 2** into the left masseter (experimental side) on the most prominent (i.e. highest) point of the muscle belly, which is approximately 2 cm above the mandibular border and 2 cm anterior of the ramus border (Svensson et al. 2003b). Immediately after the insertion of the needle used for the injections, the pain intensity was recorded with an electronic visual analogue scale (eVAS) (Shimada et al. 2015), which continued until the pain disappeared. Five minutes after the glutamate or NGF injections, the participants were asked to fill in the McGill Pain Questionnaire (MPQ) to evaluate the quality of their pain (Drewes et al. 1993). After that, measurements of PPTs, Temporal summation, and functional tests (chewing-induced pain and fatigue) were repeated. Microbiopsies were then obtained from the masseter on the contralateral side of injection.

The second session (day 7 in experiment 1 and day 3 in experiment 2) involved performance of PPT, TS, and functional tests on both sides as previously described prior to administration of injections. Subsequently NGF **(experiment 1)** or glutamate **(experiment 2)** was injected on the experimental side. **In experiment 1**, a one-week wash-out period was used to avoid any possible interaction between NGF (day 7) and the previously injected glutamate (day 0) on the muscle afferent fibres. **In experiment 2,** glutamate was injected 3 days post-NGF injection (day 0) to investigate the effect of glutamate on a muscle sensitised by NGF. In both experiments, and immediately after the injections, the pain intensity was recorded, as described. All other pain assessments were performed 5 minutes after the injections.

During the third session (day 10 in experiment 1 and day 4 in experiment 2), PPT, TS, and functional tests were carried out. Subsequently, microbiopsies were obtained from the masseter on the experimental side for later comparison with the biopsies obtained from the contralateral side during the first session.

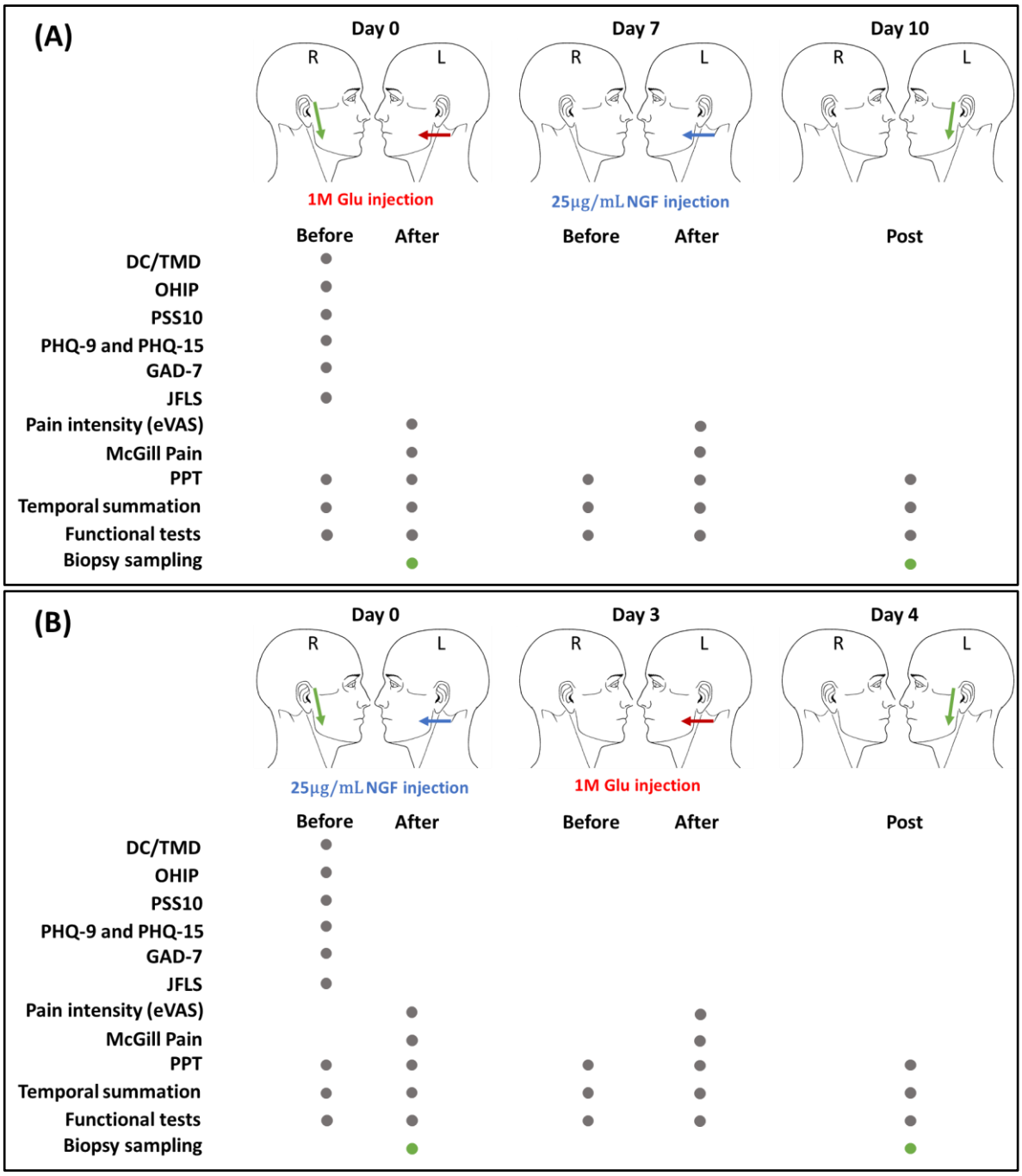

Figure 4: Presentation of the design for experiment 1 (A) and experiment 2 (B) as well as the assessments performed before and after injections in each experiment. The green arrows indicate the side in which the microbiopsies were taken. Blue arrows indicate the area of NGF injection, while red arrows indicate the area of glutamate injection.

4.3 MICROBIOPSIES

This thesis employed a novel and unique microbiopsy method developed by Christidis and co-workers in 2014 for obtaining a sufficient amount of muscle tissue with only minor inconvenience for the participant (Christidis et al. 2014). Microbiopsies with a mean weight of approximately 20 mg and a mean volume of approximately 13 mm^3 (Christidis et al. 2014) were obtained by inserting a microbiopsy instrument through the skin and into the most prominent part of the masseter muscle. After 30 minutes of skin surface anaesthesia with a

prefabricated anaesthetic patch (EMLA Patch®, 25mg lidocaine and 25 mg prilocaine, AstraZeneca, Södertälje, Sweden), a disposable coaxial biopsy needle (Bard®TruGuide™; BARD Norden, Helsingborg, Sweden) was inserted along the near long axis of the muscles until the fascia was penetrated to standardised depth of 10 mm. A disposable Monopty®Bard® biopsy instrument (18G) was passed through the coaxial needle to a penetration depth of 11 mm and used to collect the masseter muscle biopsy (Figure 5). To ensure that an equivalent region was sampled across all participants, the guiding coaxial needle was inserted at an angle of 45 degrees, 1 cm below the zygomatic arch, to a depth of 1.0 cm. This allowed the muscle biopsy to be obtained from the most prominent part of masseter muscle for each subject ***(Study I, II and IV)***.

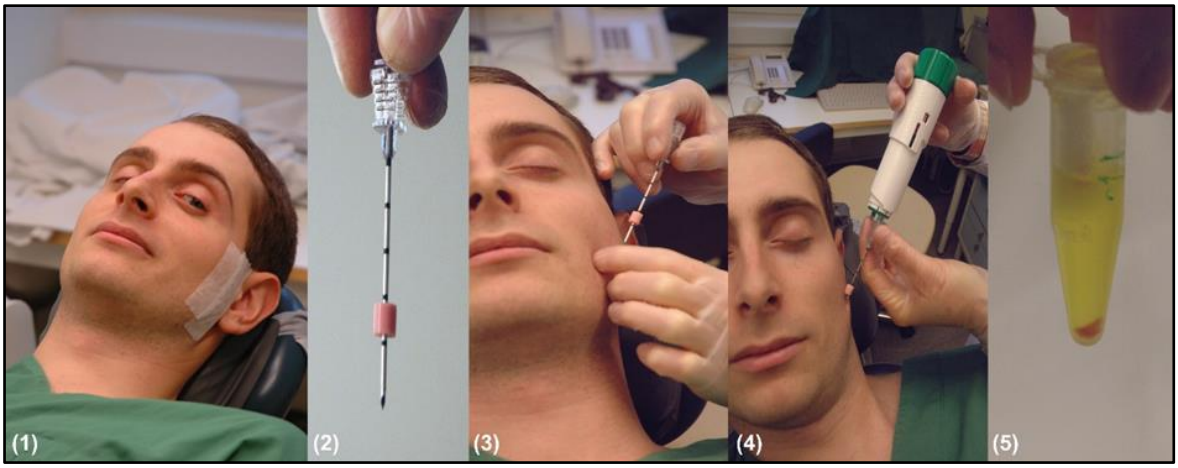

Figure 5: Topical anaesthesia (EMLA Patch®, 25mg lidocaine and 25 mg prilocaine, AstraZeneca, Södertälje, Sweden) was applied to the most prominent part of the masseter muscle for half an hour **(1)**. A co-axial needle within a guiding instrument (Bard®TruGuide™; BARD Norden, Helsingborg, Sweden) **(2)** was inserted with an angulation of 45 degrees, 1 cm below the zygomatic arch, along the near long axis of the muscles until the fascia was penetrated to marked depth of 10 mm **(3)**. The needle was then removed, while the instrument remained in place. Finally, a biopsy instrument (Monopty®Bard®) with a penetration depth of 11 mm and a diameter of 18G was inserted through the guiding instrument **(4)** to collect the masseter muscle biopsy **(5)** ***(Study I, II and IV)***.

4.4 ASSESSMENT OF EXPERIMENTALLY-INDUCED PAIN AND SENSITISATION

4.4.1 Pain intensity

Pain intensity was assessed with an electronic visual analogue scale (eVAS) ranging from 0 (no pain) to 10 (worst pain imaginable). The mean peak pain intensity over an interval of 10 minutes was calculated and used in the analyses. The MPQ is composed of 80 adjectives that relate to pain. The participants chose those adjectives that best described their pain. The adjectives are divided into sensory, affective, evaluative, and miscellaneous sections and each section is graded according to severity. The sum of the scores for the four categories can be calculated as the Pain Rating Index (PRI), which was used in this thesis (Drewes et al., 1993) ***(Study II, III and IV)***.

4.4.2 Pressure Pain Thresholds

PPT were recorded bilaterally at two points (injection point [P1] and a point 1 cm superior to the injection point [P2]) of the masseter muscle with an electronic algometer (Somedic Sales AB, Hörby, Sweden). Each recording of PPT was performed three times at each site and the mean of the three recordings was used for later analysis ***(Study II, III and IV)***.

4.4.3 Temporal summation

Temporal summation was evoked with a 1.0 kg palpometer (Palpeter®, Sunstar Suisse SA, Switzerland). The stimulus was applied 10 times at the masseter muscle (injection point [P1]) for 1 second with 2-second interval and pain intensity was recorded after the first, fifth, and tenth stimulation on a 0-10 numeric rating scale (NRS) with the same endpoints as the eVAS ***(Study II, III and IV)***.

4.4.4 Functional tests

Participants were asked to chew gum (V6, 2 piece, Fertin Pharma, Vejle, Denmark) for 1 minute and thereafter to score the pain and fatigue felt (Häggman-Henrikson and Eriksson, 2004). The pain intensity was assessed with 0-10 NRS and the fatigue level with Borg's Rating of Perceived Exertion scale (RPE) (Borg, 1982). This scale ranges from 6 (no exertion at all) to 20 (maximal exertion) ***(Study II, III and IV)***.

4.5 IMMUNOHISTOCHEMISTRY

The microbiopsies were fixed with 4% paraformaldehyde at 4°C overnight. They were subsequently rinsed in phosphate-buffered saline (PBS), dehydrated, and frozen in a -80°C freezer until sectioned. The microbiopsies were placed in a plastic mould (Tissue-Tek disposable vinyl specimen mould, 10mm * 10mm *5mm) and embedded in optimal cutting temperature (O.C.T) compound (Fisher Scientific, Ontario, Canada). Sections were sliced using a cryotome to a thickness of 10 μm, then mounted on glass slides, and stored at 37°C overnight. The sections were incubated in normal donkey serum for 1 hour before they were incubated again for 24 hours with primary antibodies against the specific axonal marker protein gene product (PGP 9.5) (1:250; anti-PGP 9.5 antibody, ABCAM Inc, Cambridge, England; ab72911) and antibodies against the NR2B subunit (1:200; anti-NMDAR2B antibody, ABCAM Inc, Cambridge, England; ab65783), SP (1:1000; anti-SP antibody, ABCAM Inc, Cambridge, England; ab10353), and NGF (1:20; Human beta-NGF Affinity Purified Polyclonal Ab, R&D Systems Inc, 614 McKineley PL NE Minneapolis, AF-256-NA). The specificity of the antibodies was previously demonstrated (Wong et al. 2014). Sections were rinsed with PBS and incubated with fluorescent secondary antibodies (Alexa 488 donkey-anti-mouse, 1:700 for PGP 9.5, and Alexa Fluor 546 donkey anti-rabbit, 1:700 for NR2B, and Alexa Fluor 633 donkey anti-goat, 1:700 for NGF, ThermoFisher, Burlington, ON, Canada. Alexa Fluor405 donkey anti-guinea pig, 1:700 for SP, Sigma-Aldrich, MO, USA). A Leica TCS SPE Confocal Microscope (Leica microsystems, Wetzlar, Germany) was used to visualise the sections and images for analysis were captured with a Leica scanner attached to the microscope.

The omission of the primary antibodies did not result in specific staining of the tested sections ***(Study I, II and IV)***.

4.6 HEMATOXYLIN STAINING

Hematoxylin (HTX) staining was performed prior to the immunohistochemical analysis on a few sections to assist in the characterisation of cell types within the biopsies (Figure 6). After drying the glass-slides in a dark room at room temperature for 24 hours, they were hydrated in a tap-water bath for two min, followed by a 20 min immersion in Harris HTX-solution (Thermo Fisher Scientific, Göteborg, Sweden). The glass-slides were subsequently dipped in a solution of 70% ethanol and 0.1% hydrogen chloride, and lastly, they were rinsed in a tap-water bath for an additional 2 min. The glass-slides were then dehydrated in baths of 70% and 95% ethanol for 2 min each and immersed in Technical Xylene (Avantor LLC, Radnor, PA USA) for 10 min. Finally, thin glass coverslips were mounted on each slide with Histomount (Thermo Fisher Scientific, Göteborg, Sweden) and micrographs were captured using a light microscope ***(Study I, II and IV)***.

The appearance of myocytes in sections of skeletal muscle is that of spherically- or cylindrically-shaped cells with striations and multiple nuclei (Frontera and Ochala 2015). Otherwise known as muscle fibres or muscle cells, myocytes are circumscribed by the endomysium, which is a loose connective tissue with a typical composition of large numbers of cells and loose fibre organisation. Meanwhile, the perimysium is the layer of connective tissue that envelops muscle fibre bundles and is made up of the majority of nerve fibres and blood vessels (Corcoran and Goldman 2020). Histological characteristics were used in this work to distinguish between the examined tissues (Corcoran and Goldman 2020; Frontera and Ochala 2015), which enabled the differentiation of nerve fibres according to whether they were related to myocytes or connective tissue ***(Study I, II and IV)***.

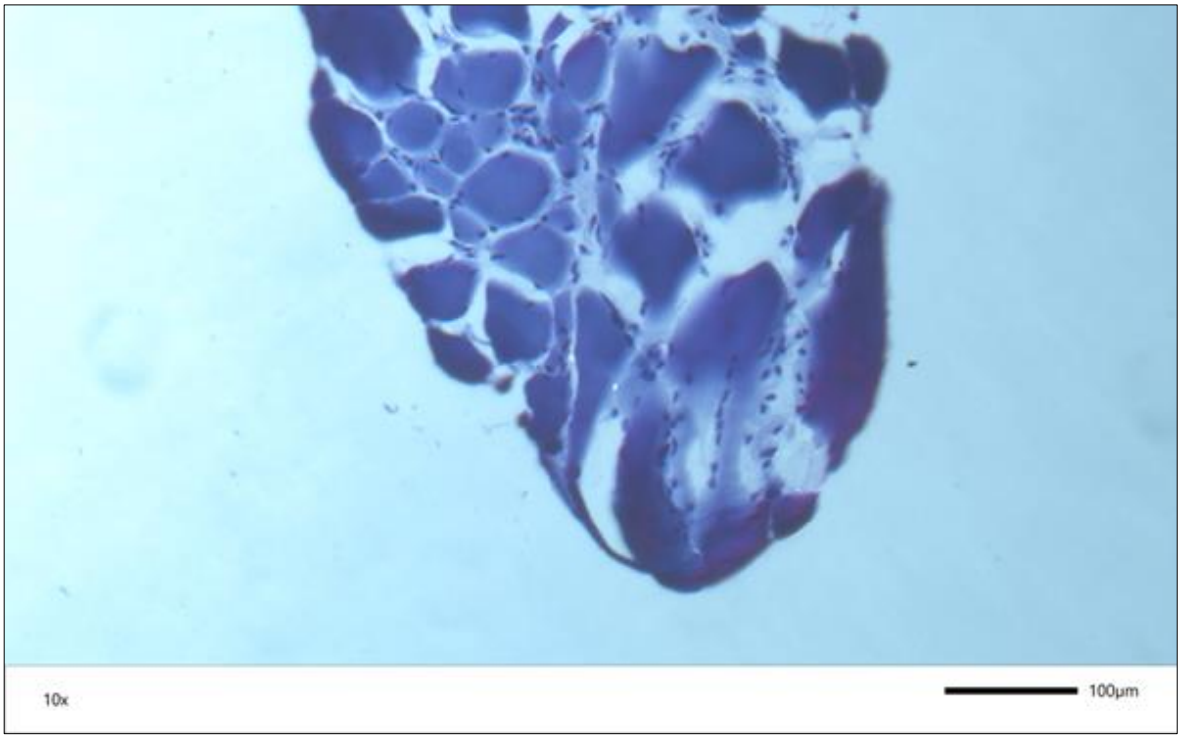

Figure 6: Illustration of high-powered (10x) photomicrographs from a single section after HTX staining from one female participant. The picture presents a group of cylindrical or round well-defined cells with multiple nuclei at the periphery (myocytes).

4.7 IMAGE ANALYSIS

Pictures were taken whenever positive nerve fibres were found under the microscope. Nerve fibres were considered positive when fluorescent signals exceeded an estimate of 95% confidence interval, i.e. the mean background of the picture + 2 SDs, and had a minimum length of 4 µm and a maximum width of 5 µm (Wong et al. 2014). Fluorescent signals separated by 5 µm or less, sharing the same path and tissue, were considered the same fibre. Using the image processing and analysis program ImageJ (Image Processing and Analysis in Java; National Institutes of Health, USA), the area (mm2) of PGP9.5 positive nerve fibres associated with myocytes or within connective tissue was calculated. Moreover, a mask for every image was created and used to count PGP 9.5 positive nerve fibres. This mask was also used to detect the colocalisation of PGP 9.5 positive nerve fibres with the different peptides of interest, namely SP, NR2B, and NGF (Figure 7) ***(Study I, II and IV)***.

SP was used as a marker to identify sensory afferent fibres. Since most of the images examined contained different amounts of myocytes and connective tissue, the density of innervation in each tissue was calculated separately to avoid bias. This was done by normalising the PGP 9.5 positive counts to the area (mm^2) of the tissue present in the images, meaning that the number of positive PGP 9.5 fibres in the tissue was divided by the total area of the same tissue on the image and finally this was averaged over the number of images for each participant. To detect if NR2B, SP, and NGF were colocalised with PGP 9.5 positive fibres, the masks for each marker were overlapped using a specific plugin within the ImageJ program known as image calculator. Overlapping signals were counted and the frequency of expression was calculated using the following equation; number of PGP 9.5 positive fibres that were colocalised with

other substances divided by the total number of PGP 9.5 positive fibres in the image averaged over the number of images for each participant ***(Study I, II and IV)***.

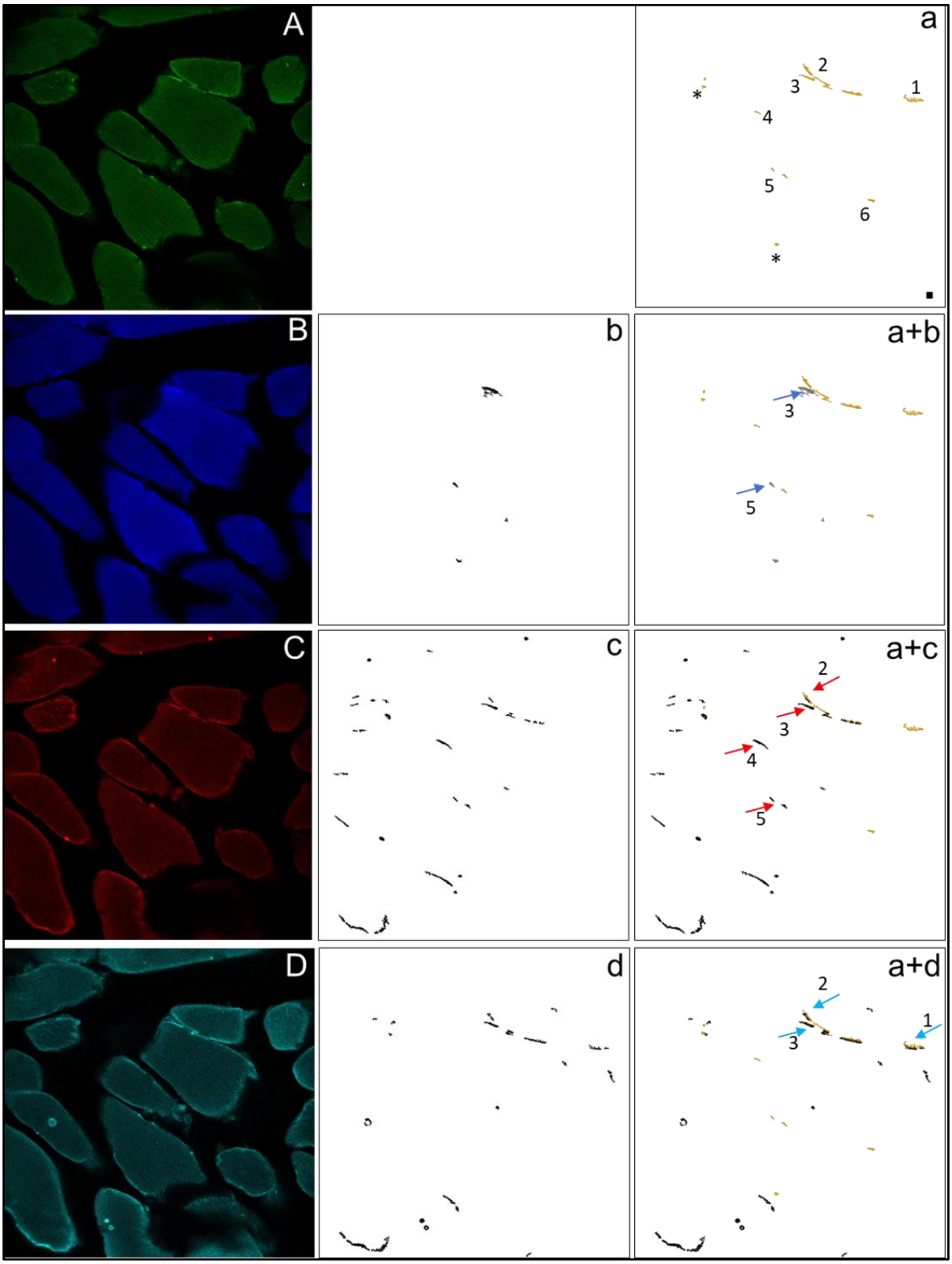

Figure 7: Examples of high-powered (40x) photomicrographs from a single section containing myocytes, from one male participant. The presented pictures show fluorescent signalling of **(A)** PGP 9.5, **(B)** SP, **(C)** NR2B, and **(D)** NGF. **(a)** the mask used for PGP 9.5 analysis, showing the number of positive PGP9.5 immunofluorescence in the section. *= indicates positive

PGP9.5 immunofluorescence; however, not counted as positive fibres as it was less than 4μm in length. Black scale square = 5 μm* 5 μm. The mask for SP is shown in **(b)**, for NR2B in **(c)**, and for NGF in **(d)**. Finally, blue, red and baby blue arrows indicate the colocalisation of positive nerve fibres with SP, NR2B, and NGF, and are presented respectively in **(a+b)**, **(a+c)** and **(a+d)**.

4.8 STATISTICAL ANALYSIS

SigmaPlot for Windows version 14.0 software (Systat Software Inc., San Jose, CA, USA) was used for all statistical analysis. In order to evaluate the normality of the data, the Shapiro-Wilk test was used. Depending on the normality test, parametric or non-parametric tests were used. Descriptive data are presented as mean ± standard deviation (SD) or median (IQR) depending on the distribution. The significance level was set at $P < 0.05$ for all analyses.

4.8.1 Experimentally-induced pain and sensitisation

In *Studies II and III*, the difference in the mean peak pain intensity, as well as the median PRI scores (MPQ) between injections (glutamate and NGF), was analysed through Paired t-tests and Wilcoxon Signed Rank Test, respectively. The sex differences in peak pain after each injection were analysed via t-tests, while the Mann-Whitney U-test was used for MPQ. Normalised PPT (Post-injection PPT data divided by the baseline data multiplied by 100) was analysed with a two-way repeated-measures (2-way RM) analysis of variance (ANOVA) with the factors of time (3 levels: experiments sessions) and sex (2 levels: men and women). The 2-way RM ANOVA was followed by post-hoc comparisons with the use of the Holm-Sidak method. For the other parameters (temporal summation, chewing pain, and fatigue), data were not normally distributed, so RM ANOVA on Ranks, factor: time, with Tukey-test as a post-hoc test was used, and the Mann-Whitney U-test was used to test sex differences. Although non-parametric tests were used, data in figures are presented as mean (SD) for better visualisation. It was determined that groups of 12 or more were sufficient to identify significant sex-related differences in experimental pain (Cairns et al. 2001; Svensson et al. 2003b).

4.8.2 Immunohistochemistry

In *Study I,* the average percentage of PGP 9.5 positive fibres in different tissues was calculated by dividing the average number of positive fibres in either connective tissue or myocytes to the average total number of positive fibres multiplied by 100. The data regarding the frequency expression of SP, NR2B, and NGF alone or in different combinations (i.e. SP/NR2B; SP/NGF; NR2B/NGF; SP/NR2B/NGF) and the density of fibres were not normally distributed and are therefore presented as median and interquartile range (IQR). The Mann-Whitney U-test was used to test for significant differences between connective tissue and myocytes, and between sexes.

In *Studies II and IV,* according to the power calculation, 12 healthy participants of both sexes were needed to be included when the power (β level) was set to 0.80, and α level to 0.05 showing an estimated difference of 30% and a standard deviation of 25% in nerve fibre

expression. Only participants whose biopsies contained the same tissue (myocytes or connective tissue) on both days were included in the analysis (Table 2).

Table 2: The number of participants whose biopsies contained the same tissue (connective tissue or myocytes) on both days when taking biopsies (day 0 and day 10 for ***Study II*** and day 0 and 4 for ***Study IV***) from ***Studies II*** *and* ***IV***. Moreover, it shows the number of participants whose biopsies contained myocytes or connective tissue in each day separately, regardless of its coexistence on both days for ***Studies II and IV***.

	Study II		***Study IV***	
	Men	**Women**	**Men**	**Women**
Participants with same tissue on both days	**Day 0 and 10**		**Day 0 and 4**	
Myocytes	5	2	8	8
Connective tissue	11	13	10	14
Participants with tissues regardless of co-existence	**Day 0**		**Day 0**	
Myocytes	8	2	8	8
Connective tissue	11	14	12	14
Participants with tissues regardless of co-existence	**Day 10**		**Day 4**	
Myocytes	9	10	10	13
Connective tissue	13	13	13	15

Significant differences and interaction between factors (sex and day) in the density and expression frequency of nerve fibres were detected by using a parametric 2-way RM ANOVA test; this was followed by post-hoc comparisons using the Holm-Sidak method. Due to the lack of samples containing myocytes in women during the first session of ***Study II***, sex differences for myocytes are not reported in that study. Pearson or Spearman correlations (depending on whether data were normally distributed) were used to examine the relationship between: **1)** the peak pain intensity 5 min after glutamate injection on day 0 **(*Study II*)** and on day 3 **(*Study IV*)**

and the expression frequency of NR2B by putative afferent fibres; **2)** the relative change (from day 0 to day 10) in mechanical sensitivity parameters (PPT, temporal summation, and chewing pain) and the relative change (from day 0 to day 10) in nerve fibre expression frequency of SP, NR2B, and NGF alone or in combination **(*Study II*)**; **3)** the relative change (from day 0 to 5 min after glutamate injection) in mechanical sensitivity parameters and the expression frequency of NR2B by nociceptive fibres on day 0 **(*Study II*)**; and **4)** the relative change (from day 0 to day 3 post-glutamate) in mechanical sensitivity parameters and the expression frequency of NGF and NR2B by putative afferent fibres on day 4 **(*Study IV*)**.

4.9 ETHICAL CONSIDERATIONS

All studies included in the thesis followed the four cardinal principles for medical research, in accordance with the Declaration of Helsinki (Avasthi et al. 2013; Shrestha 2012). The participants received clear written and verbal information, moreover, they gave their written consent before inclusion and their participation was entirely voluntary. For every study conducted as part of this thesis, ethical approval was secured from the Danish Midtjylland region on 27th August, 2015.

The microbiopsy technique used was non-invasive and painless. The size of the needle (18G) used to penetrate the skin and muscle tissue was almost similar to those used when taking blood samples (21G). The risk of side-effects from microbiopsies is very rare; the only common symptom that participants feel is a slight transient pain when the needle is inserted into the muscle. A topical anaesthetic cream is always applied to the area to reduce this discomfort as much as possible. There were no drop-outs due to pain or side-effects, only missing data due to methodological limitations. In just two cases, participants had postoperative inflammation. In such cases, anti-inflammatory medication such as Naproxen is advisable. Participants were instructed to contact the researcher anytime in case of any complications.

The quality of life of individuals suffering from TMD is negatively affected (Bitiniene et al. 2018). Furthermore, there is no solid evidence related to the aetiology of TMD myalgia. The thesis results will increase the knowledge of the peripheral mechanisms underlying musculoskeletal pain, offering new targets for new therapeutic approaches, hopefully leading to better, earlier treatments for these pain conditions thereby reducing the individual pain, suffering, and use of analgesics. Therefore, the presented thesis risks are judged to be smaller than the possible benefit to society.

5 RESULTS AND DISCUSSION

5.1 THE EFFECT OF EXPERIMENTAL PAIN MODELS ON PAIN PERCEPTION AND MECHANICAL SENSITISATION

5.1.1 Results regarding pain intensity

In both experiments **(*Studies II and III*)**, the peak pain intensity and PRI score of MPQ after injecting glutamate were significantly greater than after injecting the muscle with NGF ($P<0.001$). For all injections, no sex differences in pain intensities were detected ($P>0.05$). The peak pain intensity and PRI scores of MPQ for ***Studies II and III*** are presented in Table 3.

Table 3: The mean (SD) peak pain intensity and median (IQR) total PRI scores evoked by injections of glutamate or NGF (***Study II***), as well as of glutamate on muscle sensitised by NGF (***Study III***). A comparison in pain-related response between injections and between sexes within each study is presented.

	Study II		***Study III***	
Peak pain (eVAS)				
	Glut (day0)	**NGF (day7)**	**NGF (day0)**	**Glut (day3)**
All	6 (2)*	1 (1)	0 (1)	6 (1)*
Men	6 (2)*	1 (1)	0 (1)	6 (1)*
Women	5 (2)*	1 (1)	0 (1)	7 (1)*
MPQ total (PRI)				
All	10 (6)*	0 (0)	0 (0)	17 (14)*
Men	10 (14)*	0 (2)	0 (0)	18 (14)*
Women	10 (5)*	0 (0)	0 (0)	15 (13)*

NGF = nerve growth factor; Glut = Glutamate; MPQ = McGill Pain Questionnaire; PRI = Pain Rating Index

*= significant difference between injections (Paired t-test, $P<0.001$).

5.1.2 Results regarding pressure pain threshold (PPT)

In both experiments (on the experimental side), the injection of NGF decreased PPT significantly three days post-injection, namely, 59% decrease to before injection on day 7 **(*Study II*)** and 58% decrease to before injection on day 0 **(*Study III*)**. However, neither glutamate nor NGF significantly changed PPT (from before to 5 min after injection) during the first session of the experiments (P>0.05). **In *Study III***, PPT scores 5 min after injection of glutamate on day 3 as well as on day 4 did not change compared to before the injection on day 3 (P>0.05). For both experiments, no sex-related differences were detected at any time point except for the third session in the first experiment **(*Study II*)**, where the PPT values in women decreased significantly more than in the men (P=0.015) (Figure 8). Moreover, no significant changes were found on the control side (P>0.05).

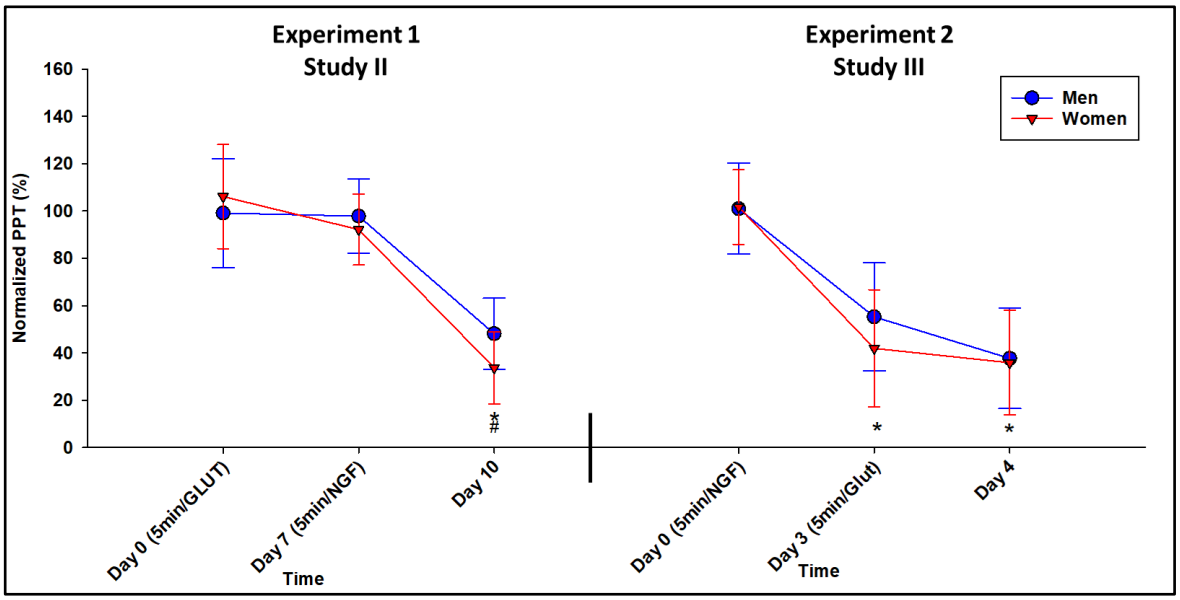

Figure 8: The PPT scores from men and women at different time points from both experiments. The change in PPT is presented normalised to before glutamate injection **(*Study II*)** and to before NGF injection **(*Study III*)**. Bars represent mean and whiskers standard deviation (SD). In experiment 1 **(*Study II*)**, the asterisk (*) indicates a significant difference from other time points (P<0.050), while in experiment 2 **(*Study III*)**, it indicates significant difference from day 0.
For both experiments: # = significant difference between sexes (P<0.050); NGF = nerve growth factor; 5min/Glut = 5 min after glutamate injection; and 5min/NGF = 5 min after NGF injection.

5.1.3 Results regarding temporal summation

In the two experiments, results indicated that the pain intensity felt by participants three days post-NGF injection was markedly higher on the experimental side during temporal summation by contrast to: **1)** before and 5 min after NGF injection (day 7 in ***Study II*** and day 0 in ***Study III***) and **2)** before and 5 min after glutamate injection **(*Study II*)** (P<0.05). **In *Study III***, pain intensity produced by temporal summation 5 min after injection of glutamate as well as on day 4 did not change compared to before the injection on day 3 (P>0.05). In both experiments, the pain intensity stemming from temporal summation was similar between the sexes at all time-

points (P>0.05), as shown in Figure 9. Likewise, the control side did not exhibit substantial alterations (P>0.05).

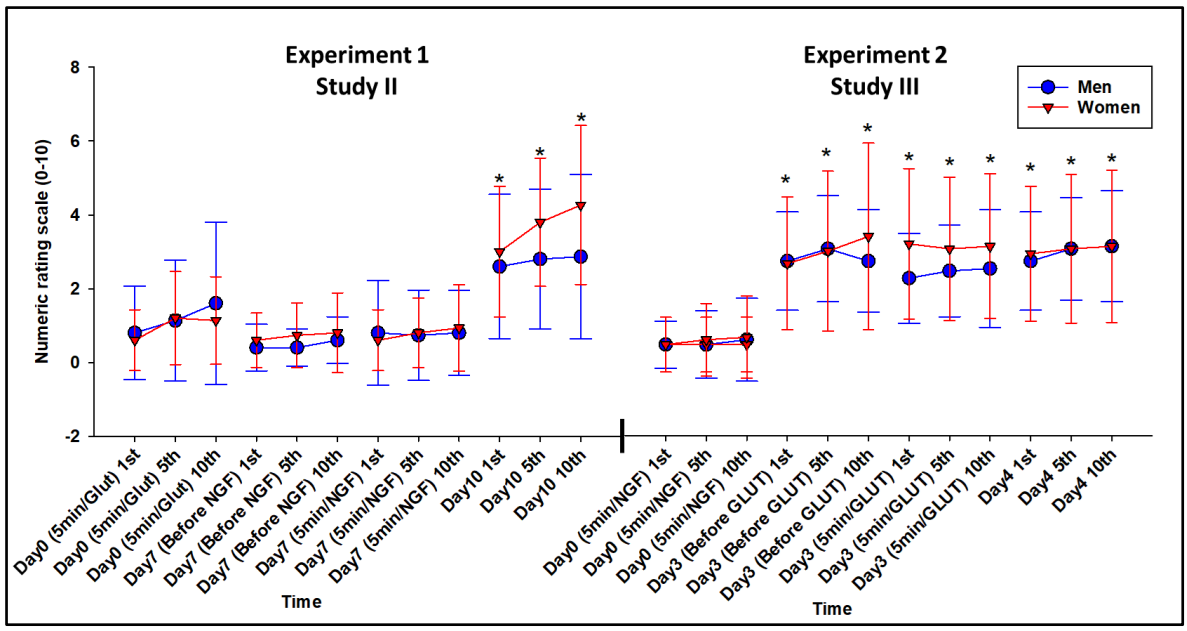

Figure 9: The mean (SD) pain ratings (NRS 0-10) for the 1st, 5th, and 10th pressure stimuli applied on men and women during a temporal summation test at different time points in two different experiments.
In experiment 1, the asterisk (*) indicates a significant difference from other time points (P<0.050), while in experiment 2 it indicates significant difference from day 0. For both experiments: NGF = nerve growth factor; 5min/Glut = 5 min after glutamate injection; and 5min/NGF = 5 min after NGF injection.

5.1.4 Results regarding chewing tests

Pain intensity and fatigue in the context of the chewing test, both increased markedly three days post-NGF injection on the experimental side by comparison with: **1)** before and 5 min after NGF injection (day 7 in ***Study II*** and day 0 in ***Study III***) and **2)** before and 5 min after glutamate injection **(*Study II*)** (P<0.05). **In *Study III***, pain intensity and fatigue induced by chewing 5 min after injection of glutamate as well as on day 4 did not change compared to before the injection on day 3 (P>0.05). The male and female participants reported comparable pain intensity and fatigue due to chewing, apart from day 10 in ***Study II*** and on day 3 five minutes post-glutamate injection in ***Study III***, when female participants scored higher than male participants in terms of pain intensity and fatigue (P<0.05), as shown in Figure 10A and B. Meanwhile, the control side did not exhibit any marked alterations (P>0.05).

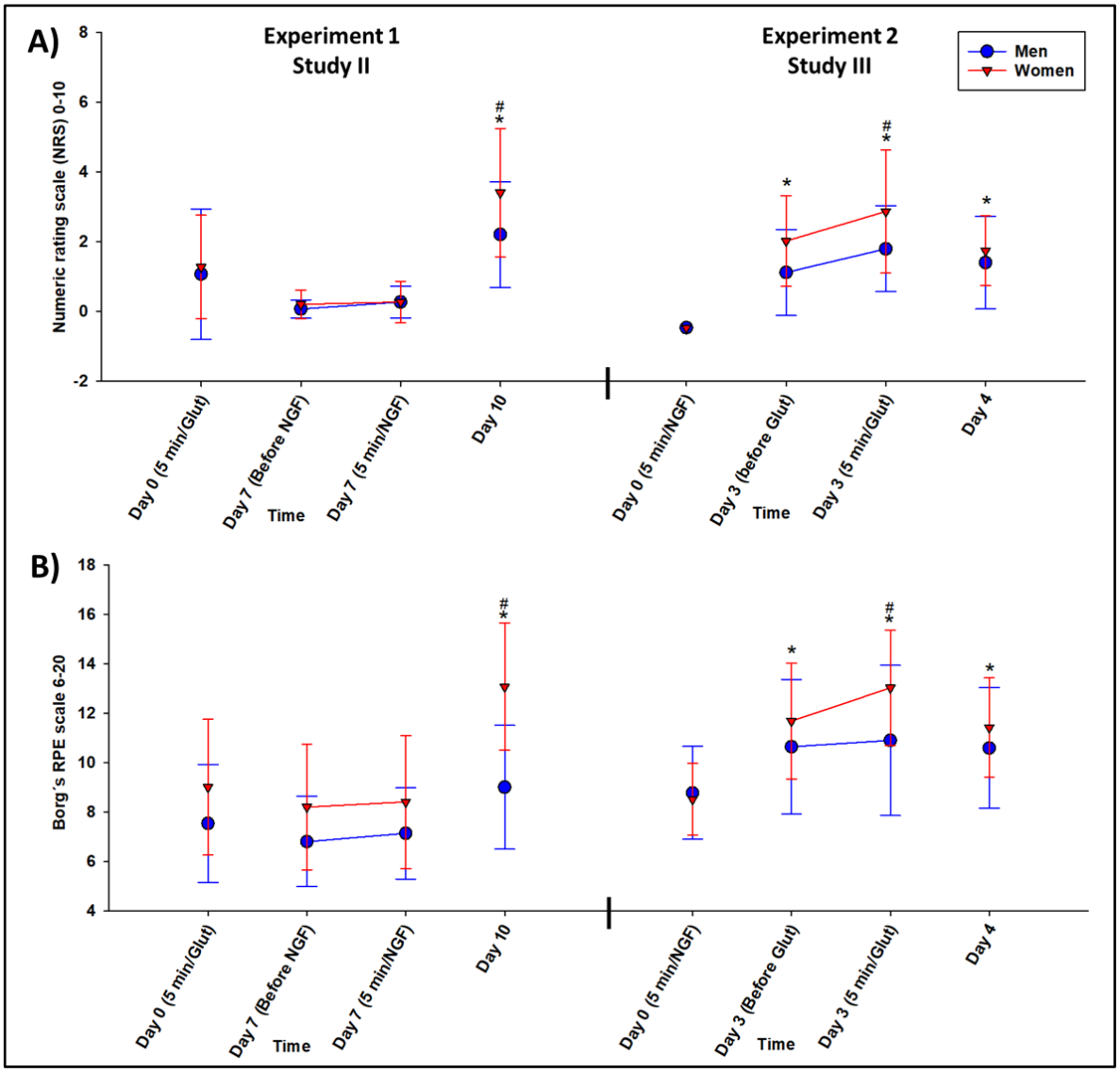

Figure 10: The mean (SD) **A)** pain ratings (NRS 0-10) and **B)** fatigue (Borg's RPE scale) evoked by a 1-min chewing test in men and women at different time points and in different experiments.
In experiment 1, the asterisk (*) indicates a significant difference from other time points (P<0.050), while in experiment 2 it indicates significant difference from day 0. For both experiments: # = significant difference between sexes (P<0.050); NGF = nerve growth factor; 5min/Glut = 5 min after glutamate injection; and 5min/NGF = 5 min after NGF injection.

5.1.5 Discussion regarding the effect of experimental pain models on pain perception and mechanical sensitisation

The pain intensity scores (eVAS) after injection of either NGF or glutamate in the current thesis **(*Study II*)** were similar to those previously reported (Svensson et al. 2003a; Svensson et al. 2003b; Svensson et al. 2008a), with glutamate injection being observed to cause pain of high intensity, while NGF injection caused only low-intensity pain. The results of the current thesis also indicated that low levels of PPT as well as high level of temporal summation pain and muscle pain and fatigue induced by chewing were experienced three days after injection of NGF (*Studies II and III*). This effect of NGF-induced muscle mechanical sensitisation reported in humans in the present thesis agrees with previous studies (Svensson et al. 2003a; Svensson et al. 2008a).

The current thesis **(*Study II*)** showed that glutamate did not affect mechanical muscle sensitisation, which contradicts previous results (Cairns et al. 2006; Svensson et al. 2003b). This difference in findings may be attributed to sensitisation induced by repeated injections of glutamate solutions compared to the single injection used in ***Study II***. Therefore, single injection may have less chance of producing significant mechanical sensitisation.

The current results show that glutamate injection into healthy human masseter muscle three days post-NGF injection does not induce: **1)** any additional pain besides the usual pain induced by glutamate (inferred by indirect comparison of the data in Table 3), and **2)** does not produce an additional effect on muscle sensitisation besides that already induced by NGF **(*Study III*)**. An earlier study produced similar results, with no change in PPT being reported when glutamate was injected one day after masseter muscle sensitisation (Svensson et al. 2008b). These findings suggest there may be a ceiling effect on sensitisation induced by NGF, which prevents further sensitisation by glutamate. Be that as it may, participants with myofascial pain had higher glutamate levels in the masseter muscle compared to participants without pain, suggesting that glutamate is among the main substances for comprehension of myalgia pathophysiology (Castrillon et al. 2010; Jasim et al. 2020).

5.2 NERVE FIBRE DENSITY AND EXPRESSION IN HEALTHY HUMAN MUSCLE

Each participant was found to have 25.3 (± 7.6) positive nerve fibres (PGP 9.5 immunoreactive fibres) on average and the majority of those fibres (72.6%) were located within the connective tissue, with a smaller proportion (27.4%) being associated with myocytes.

5.2.1 Results regarding differences between tissues within muscle

Nerve fibres within connective tissues showed greater density than nerve fibres associated with myocytes ($P < 0.001$). The median IQR nerve fibre density (fibres/area in mm^2) within connective tissue was 404 (241), while the density of nerve fibres associated with myocytes was 227 (136). Fibres within connective tissue expressed SP alone and in combination with NR2B significantly more than nerve fibres associated with myocytes ($P<0.001$). However, the nerve fibres associated with myocytes expressed NR2B and NGF alone and in combination significantly more than nerve fibres within connective tissue ($P<0.001$) (Table 4). No significant differences were detected when the co-expression of SP/NGF and SP/NR2B/NGF by nerve fibres was compared between tissues (Table 4).

Table 4: The frequency of positive fibres expressing SP, NR2B, and NGF either on their own or together in nerve fibres within connective tissue and nerve fibres associated with myocytes. Represented as median (IQR), the data derive from the participants with biopsies consisting of myocytes and/or connective tissue at day 0 in both experiments.

	Expression of nerve fibres associated with myocytes						
	SP	**NR2B**	**NGF**	**SP/NR2B**	**SP/NGF**	**NR2B/NGF**	**SP/NR2B/ NGF**
All participants (n=26)	11.4% (18.0)	91.4%# (11.0)	83.3%# (16.7)	8.8% (18.6)	8.9% (18.4)	81.0%# (19.1)	8.1% (21.3)
	Expression of nerve fibres within connective tissue						
	SP	**NR2B**	**NGF**	**SP/NR2B**	**SP/NGF**	**NR2B/NGF**	**SP/NR2B/ NGF**
All participants (n=54)	57.6%# (22.9)	44.9% (26.3)	17.1% (20.2)	29.1%# (24.7)	9.2% (10.7)	13.8% (16.5)	8.0% (10.3)

 # = Significant differences between tissues (Mann-Whitney U-test; $P < 0.05$).
n = number of participants included in the analysis.
IQR = Interquartile range (75 percentile minus 25 percentile)**.**
SP = Substance P; NR2B = NMDA receptor subunit type 2B; and NGF = Nerve growth factor

5.2.2 **Results regarding sex-related differences**

There were no significant differences between sexes concerning the density of nerve fibres. However, with regard to the expression of the nerve fibres, women displayed greater expression of SP (P=0.024), SP/NR2B (P=0.018), SP/NGF (P=0.039), and SP/NR2B/NGF (P=0.045) than men (data combined from myocytes and connective tissue) (Figure 11).

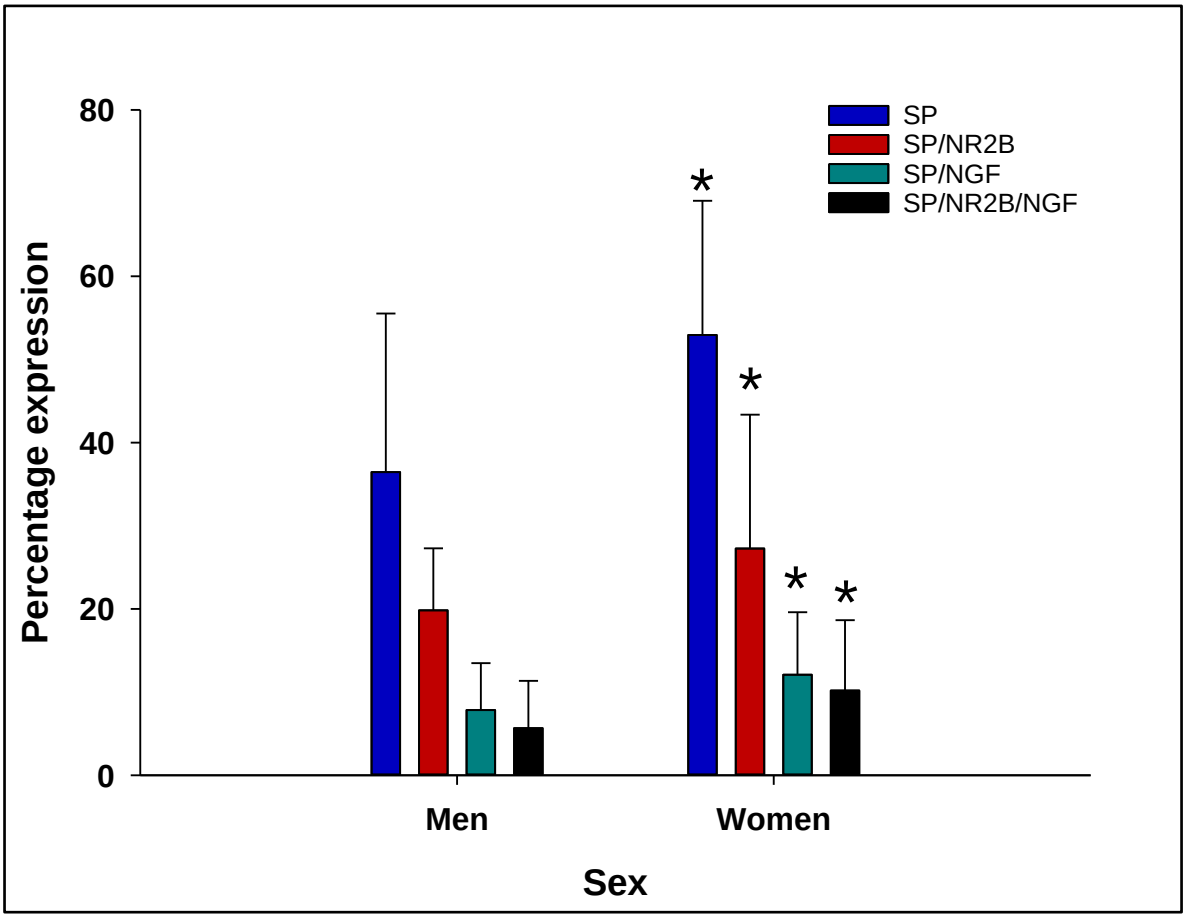

Figure 11: The median (IQR) percentage expression of SP (blue), SP/NR2B (red), SP/NGF (baby blue), and SP/NR2B/NGF (black) by nerve fibres in healthy masseter muscle tissues from men and women.
* = significant differences between sexes (Mann-Whitney U-test; $P < 0.05$)
SP = Substance P; NR2B = NMDA receptor subunit type 2B; and NGF = Nerve growth factor

5.2.3 Discussion regarding nerve fibre density and expression in healthy human muscle

A previous study showed that human masseter muscle neurons expressed NR2B and SP at a comparable level to rats (Wong et al. 2014). In that study, 44% of the positive nerve fibres expressed NR2B, while 16% expressed SP in the human masseter muscle. In agreement with that study, pooled data (without differentiating the nerve fibres within connective tissue from nerve fibres associated with myocytes) from the current thesis **(*Study I*)**, revealed nerve fibre expression of NR2B and SP by 60% and 48%, respectively. The higher expression frequency compared to the study undertaken by Wong and co-workers in 2014 may be due to the number of participants involved – the latter had 17 participants, while this study had 60 participants. However, when data from connective tissue and myocytes were analysed separately in the present thesis, the expression frequencies (Table 4) differed completely from those reported by Wong and co-workers as well as from our pooled data. Since the current results show that nerve fibres within connective tissues or nerve fibres associated with myocytes are uniquely expressing SP, NR2B, and NGF, it may be argued that the risk of one tissue affecting the other may be averted through separate analysis of the nerve fibres in every tissue of the masseter

muscle. For example, supposing samples have more myocytes than connective tissue, the results of the data will be biased to whatever the nerve fibres associated with myocytes are expressing. Therefore, it is advised to examine tissues within the muscle separately. In this way, better internal validity can be achieved in future research concerned with analysis of biomarker expression in the masseter muscle.

In a quantitative electron microscopic study performed on sensory nerve fibres of cat tibialis muscle, 34% of non-myelinated (slow conducting) fibres were nociceptive and were mostly detected in connective tissue (Stacey 1969). In agreement with animal studies, the results of the thesis **(*Study I*)** showed that nerve fibres expressing SP were mostly found within connective tissue. Furthermore, SP is considered a reliable marker for identifying non-myelinated sensory nerve fibres (Bae et al. 2015). Therefore, this supports the possibility that the nerve fibres in the connective tissue are of a sensory nature, i.e. nociceptive, compared to the nerve fibres associated with myocytes, which have shown less expression of SP by the nerve fibres **(*Study I*)**. This, on the other hand, suggests that many of the nerve fibres associated with myocytes are motor somatic efferents. These myocyte-related nerve fibres exhibited a high expression of NGF **(*Study I*)**, and hence could be a source of exogenous NGF in the masseter muscle, which in turn maintains the viability of sensory nerve fibres (Aloe et al. 2015). Moreover, the higher expression of NR2B by nerve fibres associated with myocytes **(*Study I*)** possibly reflects the role of NMDA receptors in synaptic transmission to oral-motor circuitry (Turman et al. 1999).

5.3 NERVE FIBRE DENSITY AND EXPRESSION AFTER EXPERIMENTALLY-INDUCED MYALGIA

5.3.1 Results regarding the density of nerve fibres

When NGF was injected alone on day 7 in ***Study II***, the average density of nerve fibres as well as the average density of putative afferent fibres (nerve fibres expressing SP), did not change significantly between days 0 and 10 in neither myocytes nor connective tissue. However, when glutamate was injected 3 days post-NGF in ***Study IV***, the average density of putative afferent fibres associated with myocytes increased significantly on day 4 compared to day 0 ($F=6.970$, $P=0.019$). The mean (SD) putative afferent fibre density (fibres/area in mm^2) on day 0 was 42 (33), while on day 4 it was 76 (51).

No sex-related differences were detected between the male and female participants either when NGF was injected alone **(*Study II*)** or in combination with glutamate **(*Study IV*)**.

No significant interaction between factors ($P>0.05$) was detected.

5.3.2 Results regarding the expression of nerve fibres

In *Study II* (injection of NGF alone), there were no significant differences between days 0 and 10 or sex-related differences in the expression frequency of markers (NR2B, SP, NGF) by nerve fibres within connective tissue or by nerve fibres associated with myocytes. The factors did not interact with one another significantly (P>0.05).

In *Study IV* (NGF combined with glutamate), the expression frequency of nerve fibres within myocytes to SP alone (F=13.713, P=0.002), SP/NR2B (F=10.599, P=0.006) and SP/NGF (F=5.151, P=0.040), as well as SP/NR2/NGF (F=4.774, P=0.046), was significantly greater on day 4 compared to day 0 (Figure 12A). No sex-related differences were found in nerve fibres within myocytes. Although no significant differences in the expression frequency were detected between day 0 and day 4 in nerve fibres within the connective tissue, significant differences between sexes were detected in those fibres. The expression frequency of nerve fibres to SP (F=6.296, P=0.020), NR2B (F=4.956, P=0.037), SP/NR2B (F=8.366, P=0.008), SP/NGF (F=4.375, P=0.048), and SP/NR2B/NGF (F=4.716, P=0.041) was significantly higher in women when compared with men (Figure 12B).

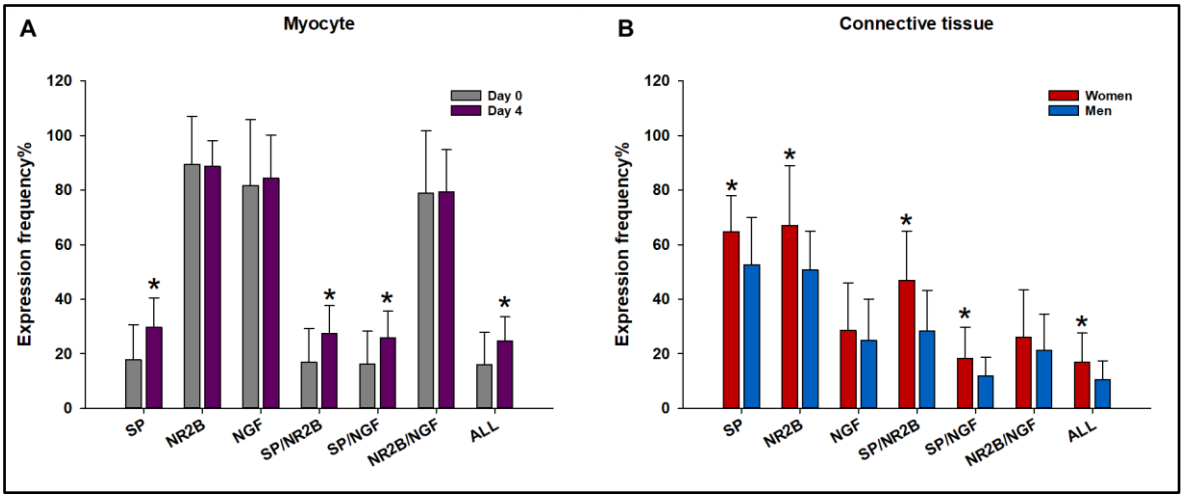

Figure 12: The difference in the expression frequency of different substances alone and in combination between days in nerve fibres associated with myocytes **(A)**, and between sexes in nerve fibres within connective tissue **(B)**. Mean (SD) values are presented. The special character * indicates significant differences between days or sexes (P < 0.05).
SP = Substance P; NR2B = NMDA receptor subunit type 2B; and NGF = Nerve growth factor

Neither data from nerve fibres associated with myocytes nor nerve fibres within connective tissue showed a significant interaction between factors (P>0.05) **(*Study IV*)**. However, a post-hoc test showed that nerve fibres associated with myocytes in women expressed SP (P=0.006) and SP/NR2B (P=0.016) significantly more on day 4 compared to day 0, but this was not found in men (P>0.05). Moreover, a post-hoc test showed that nerve fibres within connective tissue expressed SP/NR2B significantly more in women than men on day 0 (P=0.012) and on day 4 (P=0.015).

5.3.3 Discussion regarding the nerve fibre density and expression after experimentally-induced myalgia

To the best of my knowledge, most of the studies investigating changes in nerve fibre density focused on the skin and only one study focused on muscle tissue. The latter demonstrated that the density of SP-expressing nerve fibres in muscle myocytes and connective tissue was not enhanced when an inflammatory response was triggered experimentally in rat soleus and gastrocnemius muscles. However, in the same study, the density of perivascular fibres within the same muscle was significantly increased (Reinert et al. 1998). In accordance with Reinert and co-workers (1998), the current thesis **(*Study II*)** also presented no changes in the density of putative afferent fibres (expressing SP) examined in myocytes or connective tissue of the masseter muscle post-NGF injection. A debate is ongoing in the literature regarding the changes applied to sensory nerve fibre density due to mechanical sensitisation induced by NGF (Hirth et al. 2013) or due to other factors such as inflammation (Reinert et al. 1998), injury to the nerve fibres (Yen et al. 2006) or neuropathy (Cheng et al. 2012). Some studies reported increases in the density (Cheng et al. 2012; Yen et al. 2006), while others showed no changes applied to the nerve fibre density (Hirth et al. 2013; Reinert et al. 1998). However, several factors can potentially contribute to the diversity of results reported by those studies, such as: **1)** the place where the nerve fibres were examined, for example, muscle tissues (connective tissue, myocytes, and blood vessels) or skin (dermal or epidermal); **2)** the method used to identify nerve fibres, like ankyrin B or PGP 9.5; **3)** the peptides examined, such as SP and/or calcitonin gene-related peptide (CGRP); and **4)** the experimental model used to study the change in density (NGF injection, inflammation or neuropathy).

Several mechanisms have been proposed to underpin mechanical muscle sensitisation induced by NGF or glutamate. In female rats, for example, NGF-induced hyperalgesia and allodynia to the masseter muscle increased nerve fibres expressing SP by ganglion neurons (Wong et al. 2014), partly through the high-affinity tyrosine kinase receptor A (TrkA) (Svensson et al. 2010). Meanwhile, evidence from other research suggested that NMDA receptor activation partially underpinned muscle sensitisation by NGF or glutamate injection, from which it was deduced that NGF and NMDA receptors interacted with one another (Cairns et al. 2003; Wong et al. 2014). The findings from the current thesis **(*Study IV*)** support the existence of such interaction. In this thesis, the combined injection of NGF and glutamate did increase the expression of SP and the co-expression of SP/NR2B, SP/NGF, and SP/NR2B/NGF by the nerve fibres. Moreover, and as mentioned previously **(*Study III*)**, glutamate failed to cause any change in PPT when injected in muscle by NGF. This could imply that NGF and glutamate can share the same pathway, thus preventing additional sensitisation when these substances are used together (Svensson et al. 2008b). Collectively, these findings suggest the involvement of SP, NMDA receptors, and NGF in the pathophysiological mechanism related to NGF- and glutamate-induced muscle pain and sensitisation.

The injection of NGF alone **(*Study II*)** did not change the expression of putative afferent fibres. To the contrary, glutamate injection into the masseter muscle sensitised by NGF succeeded in increasing the expression of putative afferent fibres in the masseter muscle **(*Study IV*)**. The

current human results from this thesis are consistent with animal studies, where it has been shown that the injection of glutamate can excite and sensitise putative afferent fibres (Cairns et al. 2002; Cairns et al. 2001). Other animal studies have shown that NGF does not excite enough nociceptive afferent fibres required for pain sensation; however, it can sensitise putative afferent fibres to mechanical stimulation (Mann et al. 2006; Svensson et al. 2008b). Therefore, the combined injection of NGF and glutamate may work as a better pain model to study the pathophysiological mechanism of TMD myalgia in humans than the injection of NGF or glutamate alone.

5.4 THE CORRELATION BETWEEN THE EXPRESSION AND MECHANICAL SENSITIVITY OR PAIN

5.4.1 Results from *Study II*

1) The peak pain intensity 5 min after glutamate injection on day 0 was positively correlated with the expression of NR2B by nerve fibres associated with myocytes on day 0 (r_s=0.620, n=10, P=0.048, Spearman) (Figure 13A).

2) There were no correlations between the relative change (from day 0 to day 10) in mechanical sensitivity parameters (PPT, temporal summation, and chewing pain) and the relative change (from day 0 to day 10) in nerve fibre expression frequency of SP, NR2B, and NGF alone or in combination (P>0.05). Nevertheless, separate analysis of data related to male and female participants revealed that the relative change in PPT was markedly negatively correlated to the relative change in NR2B expressed by nerve fibres within connective tissue on its own (r_s= -0.659, P=0.013) and alongside NGF (r_s= -0.764, P=0.001).

3) There was a positive correlation between the relative change (from day 0 to 5 min after glutamate injection) in temporal summation and the expression frequency of NR2B by putative afferent fibres within connective tissue (r_s= 0.561, n=25, P=0.003) on day 0.

5.4.2 Results from *Study IV*

1) The peak pain intensity 5 min after glutamate injection (on muscle sensitised by NGF) on day 3 correlated positively with the co-expression of SP/NR2B (r=0.463, n=28, P=0.013) by nerve fibres within connective tissue on day 4 (Figure 13B).

2) There was a significant positive correlation between relative change (from day 0 to day 3 post-glutamate) in temporal summation pain and the expression frequency of SP/NR2B by nerve fibres associated with myocytes (r=0.437, n=23, P=0.036) as well as by nerve fibres within connective tissue (r=0.448, n=28, P=0.016) on day 4.

3) The expression of SP/NR2B by nerve fibres within connective tissue correlated positively with chewing-evoked pain (r_s=0.394, n=28, P=0.037).

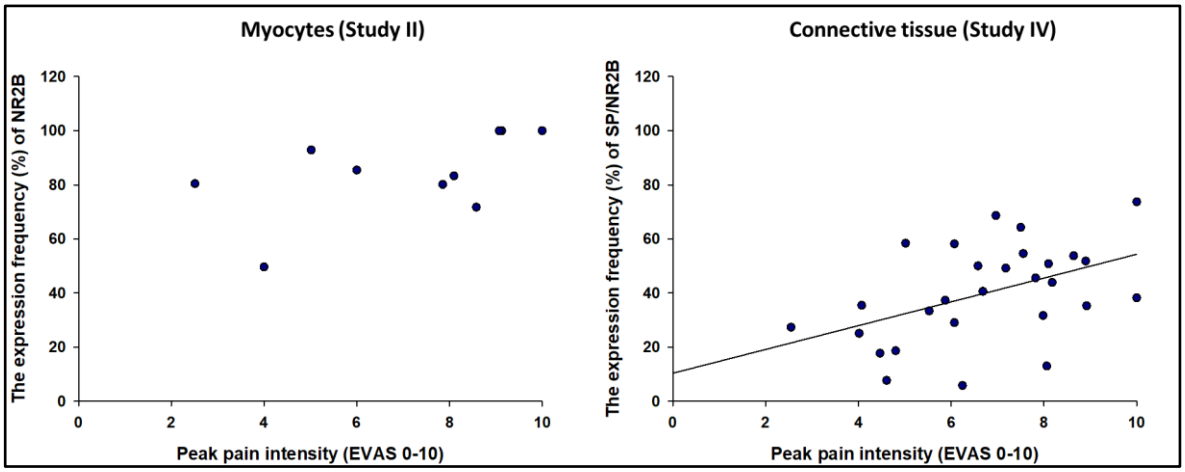

Figure 13: The correlation between **A)** the peak pain intensity 5 min after glutamate injection on day 0 ***(study II)*** and between the expression of NR2B by nerve fibres associated with myocytes on day 0. **B)** the peak pain intensity 5 min after glutamate injection on day 3 ***(study IV)*** and the co-expression of SP/NR2B by nerve fibres within connective tissue on day 4.
SP = Substance P; and NR2B = NMDA receptor subunit type 2B

5.4.3 Discussion regarding the correlation between the expression and mechanical sensitivity or pain

Masticatory muscle pain and fatigue induced by chewing are common signs of TMD myalgia (Fernández-de-las-Penas and Svensson 2016). Peak pain intensity rated post-glutamate injection into the masseter muscle was reported to be similar in healthy participants compared to the peak pain reported by individuals having TMD (Castrillon et al. 2008). Temporal summation pain in TMD individuals was shown to be higher compared to healthy individuals (Sarlani et al. 2007; Sarlani et al. 2004). In an experimental pain model, temporal summation was shown to reflect central sensitisation that was dependent on the activation of NMDA receptors (Eide 2000). There is evidence that glutamate injection into the masseter muscle is accompanied by pain and, by activating NMDA receptors, it causes excitation and sensitisation of presumptive afferent fibres (Cairns et al. 2002; Cairns et al. 2001). NGF injection into rat masseter muscle does not elicit pain, but it does sensitise muscle nerve fibres through the activation of NMDA receptors (Wong et al. 2014). The current results showed positive correlations between pain characteristics (i.e. similar to pain complaints by individuals suffering from TMD myalgia) and the expression of NMDA receptors by nerve fibres. Together, these findings further strengthen the hypothesis that the expression of NMDA receptors by sensory nerve fibres can play a part in the pathogenesis of TMD myalgia (Cairns et al. 2007; Cairns et al. 2006; Cairns et al. 2003). Hence, the masseter muscle of individuals having a higher expression of NMDA receptors may be more sensitive to pain.

5.5 SEX DIFFERENCES

The mechanical sensitisation triggered when NGF was injected into the masseter muscle was reported to persist for as many as 21 days in individuals of both sexes (Svensson et al. 2003a; Svensson et al. 2008a). Although these studies were performed on either men or women separately, the author inferred higher mechanical muscle sensitisation in women than men by indirect comparison. The current thesis **(*Study II*)** is the first to report sex differences related to NGF-induced masseter muscle sensitisation in humans. In animals, the sex differences related to muscle sensitisation by NGF have been attributed to the higher co-expression of SP/NR2B in female rats (Wong et al. 2014). Consistent with animal studies, the human findings in the thesis also showed higher baseline expression of SP/NR2B by nerve fibres in women when compared to men **(*Study I*)**. Moreover, the negative correlation found between PPT and the expression of NMDA receptors by nerve fibres in women and not in men **(*Study II*)** further supports the finding by Wong and co-workers (2014). Although no sex differences in glutamate-evoked pain were found in this thesis, unlike male participants, female participants exhibited higher pain intensity when glutamate was injected in the masseter muscle in other studies (Castrillon et al. 2012; Svensson et al. 2003b). In female rats, this has been shown to be due to the increased oestrogen levels associated with higher expression of NR2B by masseter ganglion neurons (Dong et al. 2007). Consistent with previous reports, the current results from ***Study IV*** showed that masseter muscle nerve fibres in women expressed SP/NR2B more than men post-glutamate injection. Therefore, one can postulate that the sex-related differences in glutamate-evoked muscle pain and NGF-induced muscle sensitisation are comparable in humans and rats, and thus also ascribable to the expression of NR2B by putative afferent nerve fibres.

6 GENERAL DISCUSSION

The thesis main findings were that: **1)** glutamate injection into the masseter muscle was painful but did not produce sufficient mechanical muscle sensitisation to be detected; moreover, the level of pain induced by glutamate was positively correlated with nerve fibre expression of NMDA receptors; **2)** the injection of NGF into the masseter muscle was not painful but sensitised the muscle to mechanical pressure and jaw functions; however, there were no detectable significant changes on the nerve fibres at the molecular and cellular level; **3)** the combined injection of NGF and glutamate caused pain and mechanical muscle sensitisation as well as increased the density and the expression of NR2B and NGF by putative afferent nerve fibres. The increased level of pain and mechanical sensitisation were associated with increased expression of NR2B by putative afferent nerve fibres; **4)** putative afferent nerve fibres in women demonstrated higher expression of NR2B and NGF than men. Moreover, the expression of NR2B by putative afferent nerve fibres after NGF and glutamate injections increased in women, but not in men.

TMD myalgia is frequently associated with pain (local or referred), allodynia, and hyperalgesia (Stohler 1999), which suggests nerve fibre sensitisation occurs both peripherally and centrally. Studies have identified inflammatory mediators such as histamine, bradykinin, prostaglandin and serotonin to be responsible for the peripheral afferent fibre sensitisation induced by myositis (Mense 2003). Neurotransmitters/modulators such as NGF and SP have been shown to mediate inflammatory hyperalgesia (Björkman et al. 1994; Woolf et al. 1994), with non-neuronal cells secreting NGF with the help of cytokines (Freund et al. 2002; Manni et al. 2003; März et al. 1999; Steiner et al. 1991; von Boyen et al. 2006). Furthermore, retrograde transport of NGF to the dorsal root ganglion occurs when tyrosine kinase A (TrkA) receptors and 75-kDa neurotrophin receptor ($p75^{NTR}$) are activated (Sung et al. 2018; Woolf 1996), leading to enhanced SP transcription (Goedert et al. 1981). However, as mentioned earlier fibromyalgia (Clauw and Crofford 2003; Henriksson 2003) and the majority of TMD do not show signs of inflammation (Singer and Dionne 1997; Zarb et al. 1995), which means that the receptors mechanisms underlie the occurrence of TMD myalgia is different (Lam et al. 2005).

Although inflammation is most probably not the cause of TMD myalgia (Singer and Dionne 1997; Zarb et al. 1995), different neurotransmitters and algogenic substances such as serotonin and glutamate have been shown to be elevated in TMD myalgia patients (Castrillon et al. 2010; Christidis et al. 2014; Ernberg et al. 1999). This is especially the case with the neurotransmitter glutamate, which has been demonstrated to be upregulated in the interstitial fluid of the masseter muscle, plasma, and saliva of TMD myalgia patients (Castrillon et al. 2010; Jasim et al. 2020). Moreover, glutamate and NGF levels in cerebrospinal fluid were positively correlated in fibromyalgia patients (Sarchielli et al. 2007).

Sensitisation of primary afferent fibres occurs through the activation of NMDA receptors for glutamate (Cairns et al. 2002; Cairns et al. 2006; Cairns et al. 2003), the 5-HT3 receptors for serotonin (Ernberg et al. 2000a; 2000b; Sung et al. 2008) and TrkA receptors for NGF

(Svensson et al. 2010). Direct and indirect interaction between NGF and NMDA receptors was previously reported. In rats, NMDA receptor functions in cultured hippocampal neurons were enhanced directly by NGF (Jarvis et al. 1997). An indirect effect of NGF was the activation of NMDA receptor phosphorylation (specifically NR2 A and B subunits) through the Trk receptors in the spinal cord (Di Luca et al. 2001). The injection of NGF into rat masseter muscle increased NR2B expression in the trigeminal ganglion (Wong et al. 2014). Almost 85% of the neurons expressing NR2B in the rat trigeminal ganglion also co-expressed TrkA or P75 receptors (Svensson et al. 2010).

The present thesis confirmed that SP, NR2B, and NGF at the peripheral end of masseter afferent nerve fibres were involved in the pathophysiological mechanism of experimental myalgia in humans. Moreover, it indicated the existence of a correlation between the presence of these molecules (specifically SP/NR2B) and pain, hyperalgesia, and allodynia associated with experimental myalgia. With this in mind and by gathering all previously mentioned findings, one can speculate the following mechanism for TMD myalgia. Noxious chemical or mechanical stimuli applied to the masseter muscle elevate algogenic substances (serotonin and glutamate), neuropeptides (SP and CGRP), and neurotrophins (NGF) in the area affected. In turn, these substances bind to their specific receptors on afferent nerve fibres and function as polymodal nociceptors, causing pain, allodynia, and hyperalgesia. The ongoing release of neurotransmitters in the peripheral afferents will enhance central sensitisation and lead to the development of TMD myalgia. However, to prove this hypothesis, the results of the current thesis must be translatable to TMD myalgia patients.

7 CONCLUSIONS

Pain and sensitisation akin to the clinical manifestations of TMD myalgia can be effectively triggered experimentally by injecting NGF as well as glutamate in healthy masseter muscle. It appears that both muscle sensitisation mechanisms and pain discrepancies between men and women are critically dependent on peripheral presumptive sensory afferent fibres expressing NMDA receptors and NGF within the model of masseter muscle myalgia employed in this thesis. Nevertheless, more research is needed to determine if the findings obtained are applicable to clinical muscle pain mechanisms like TMD myalgia.

8 FUTURE PERSPECTIVE

Research has already been commenced on the expression of presumptive pain biomarkers, including serotonin receptors (5-HT_3), sodium channels (Na_V1.8 and 1.7), transient receptor potential channel (TrpV1), NR2B, and SP by nerve fibres in the masseter muscle of people suffering from TMD myalgia or myofascial pain with or without referral as well as in healthy people. Future work should also address the shortcomings of methodology. For instance, muscle tissues were insufficient or entirely absent from a handful of biopsy samples, meaning that there were fewer participants in whom expression evaluation could be undertaken, leading to the possibility of distorted interpretation of data. Hence, a novel method involving the use of ultrasound imaging for better needle positioning within the muscle is being devised for muscle biopsy extraction (Figure 14).

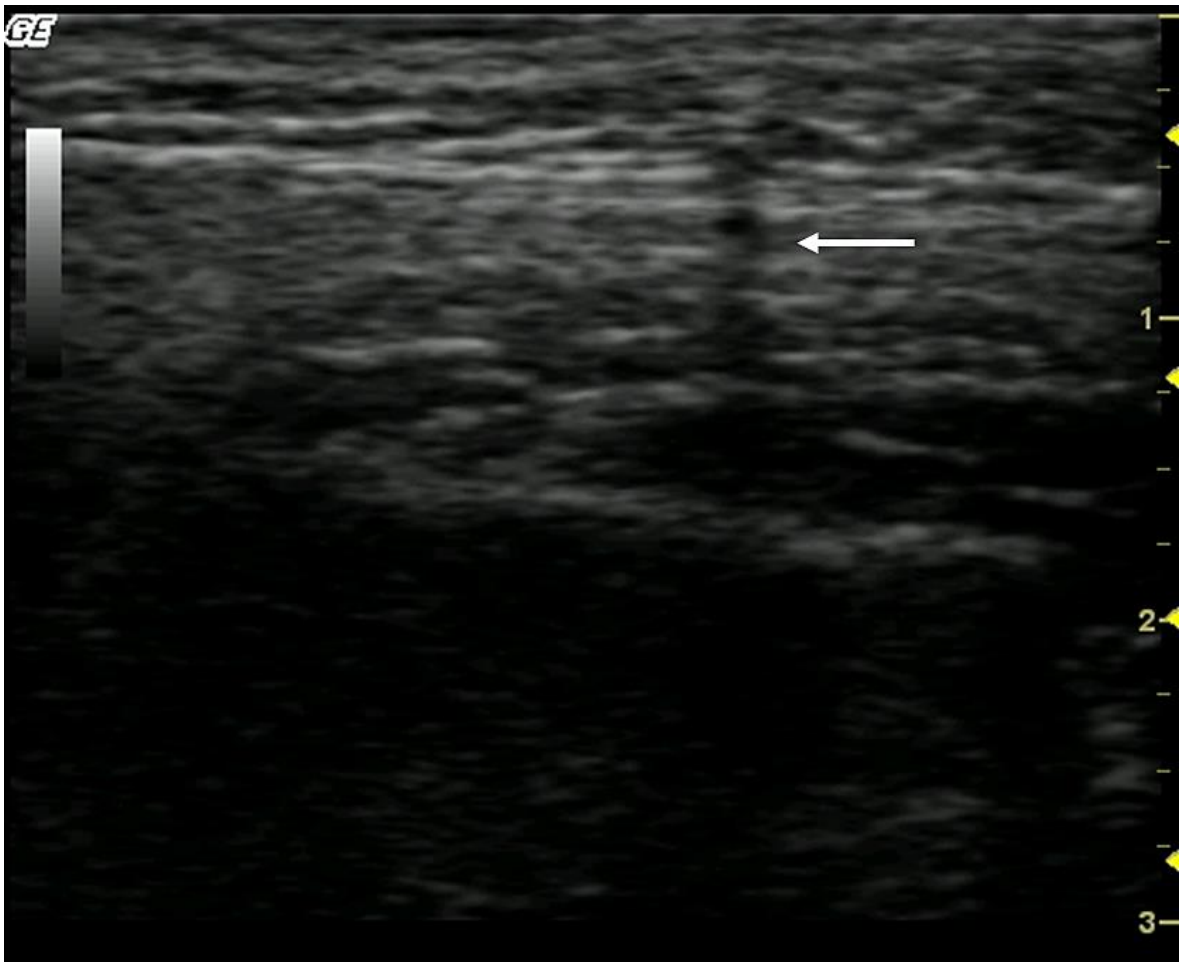

Figure 14: Illustration of ultrasound imaging of the masseter muscle from one female participant. The white arrow (dark shadow) indicates the penetration of the coaxial needle in the muscle.

The use of a single neuropeptide (SP) for detection of sensory afferent fibres constitutes a research shortcoming as well, resulting in possible underestimation of the number of muscle afferent fibres. Thus, future studies should employ additional neuropeptides, such as CGRP, as well as the sodium channels Na_V1.8 and 1.7. The use of CGRP is warranted by evidence from rat studies indicating their expression occurs in a proportion of over 60% of masseter-innervating trigeminal ganglion neurons (Iyengar et al. 2017; Wong et al. 2014).

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